

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Bell *et al.*

Confirmation No.: 4773

Appl. No. 09/664,444

Art Unit: 1645

Filed: September 18, 2000

Examiner: R. Zeman

For: **ONCOLYTIC VIRUS**

Atty. Docket: 18003

Cust. No. 31976

PATENT

Mail Stop Appeal Brief – Patents

June 15, 2009

Commissioner for Patents

P.O. Box 1450

Alexandria, VA 22313-1450

APPEAL BRIEF

Sir:

A Notice of Appeal was timely filed on January 14, 2009 in connection with the above-identified patent application. An Appeal Brief was due March 14, 2009, which is two months from the date of filing of the Notice of Appeal (37 CFR 41.37(a)(1)). Appellants hereby petition for a three-month extension of time. With the granting of the petition an Appeal Brief is now due Monday, June 15, 2009. The fee for filing an Appeal Brief (37 CFR 41.20(b)(2) and 41.37(a)(2)) and the extension of time fee are submitted herewith. Accordingly, this Appeal Brief is being timely filed.

TABLE OF CONTENTS

<u>Section</u>	<u>Page Number</u>
I. Real Party in Interest	3
II. Related Appeals and Interferences	4
III. Status of Claims	5
IV. Status of Amendments	6
V. Summary of Claimed Subject Matter	7
VI. Grounds of Rejection to be Reviewed on Appeal	8
VII. Argument	9
1. 35 U.S.C. § 112, first paragraph (Biological Deposit)	
a. Claims 27-29, 31, 73-75 and 77	9
b. Claims 30 and 76	12
2. 35 U.S.C. § 112, first paragraph, enablement	
a. Claims 1, 6-13, 19, 24-37, 64-77, 79-80	13
VIII. Conclusion	34
IX. Claims Appendix	35
X. Evidence Appendix	41
XI. Related Proceedings Appendix	45

I. REAL PARTY IN INTEREST

The real party in interest is the assignee, Wellstat Biologics Corporation.

II. RELATED APPEALS AND INTERFERENCES

To the knowledge of the undersigned, there are no appeals, interferences or judicial proceedings that are related to, would directly affect, be affected by or have a bearing on the Board's decision in the present appeal.

III. STATUS OF CLAIMS

Claims 1, 6-13, 19, 24-37, 64-77 and 79-80 have been rejected and are under appeal.

Claim 78 is objected to as being dependent on a rejected claim.

IV. STATUS OF AMENDMENTS

On September 16, 2008, Applicants submitted an Amendment under 37 CFR § 1.116 seeking solely to cancel withdrawn claims 2-4 and 14-17. Although the Advisory Action of November 3, 2008 did not explicitly indicate whether the amendment had been entered, the Notice of Panel Decision from Pre-Appeal Brief Review did not list claims 2-4 and 14-17 as being allowed, objected to, rejected or withdrawn. Applicants believe that the Amendment has been entered. Accordingly, the Claims Appendix does not include claims 2-4 and 14-17.

V. SUMMARY OF CLAIMED SUBJECT MATTER

Independent claim 1 is directed to methods of reducing the viability¹ of a hematopoietic tumor cell², comprising administering to the tumor cell a vesicular stomatitis virus³, wherein the virus is contained in a cell infected with the virus and the administering comprises administering the virus-infected cell⁴.

Independent claim 35 is directed to methods of reducing the viability of a tumor cell within a population of cells⁵ (comprising hematopoietic tumor cells and non-tumor cells) comprising administering a vesicular stomatitis virus³ to the population of cells, such that the virus is delivered to the population of cells. The virus is contained in a cell infected with the virus and the administering comprises administering the virus-infected cell⁴. The virus is able to selectively reduce the viability of the hematopoietic tumor cells².

¹ page 4, lines 6-7; page 11, lines 16-17; and original claim 1

² page 18, lines 17-20 and 25-28; page 19, lines 27-31; page 29, lines 12-16; page 41, lines 18-32; page 43, lines 20-28; page 51, lines 2-24; tables 1 and 6-7; and original claims 1-2, 5-13 and 23

³ page 4, line 10; page 11, lines 29-30; page 20, lines 1-12; page 31, line 18 to page 32, line 18; and original claims 20-22 and 26-31

⁴ page 19, lines 18-20; page 32, lines 22-28; page 33, lines 3-10; page 44, lines 12-13 and 19; figures 5-7; and original claim 34

⁵ page 4, lines 18-26; page 30, line 28 to page 31, line 16; page 50, lines 4-18; page 51, lines 2-24; and original claim 35

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The two grounds of rejection for review by the Board is whether:

(i) claims 27-31 and 73-77 are unpatentable under 35 U.S.C. § 112, first paragraph, for failing to meet the enablement requirements without a biological deposit; and

(ii) claims 1, 6-13, 19, 24-37, 64-77 and 79-80 are unpatentable under 35 U.S.C. § 112, first paragraph, for failing to meet the enablement requirements.

VII. ARGUMENT

A. Rejection under 35 U.S.C. § 112, first paragraph, for failing to meet the enablement requirements without a biological deposit

(i) Claims 27-29, 31, 73-75 and 77

Claims 27-29, 31, 73-75 and 77 remain rejected under 35 U.S.C. § 112, first paragraph, for failing to meet the enablement requirements without a biological deposit. Applicants respectfully submit that the claims satisfy the requirements of 35 U.S.C. § 112, first paragraph, and do not require a biological deposit.

The Examiner states,

[t]he deposit of biological organisms is considered by the Examiner to be necessary for the enablement of the current invention (see 37 CFR 1.808(a)).

(July 16, 2008, Office Action, page 4.) Applicants respectfully disagree.

With regards to an enablement rejection, “[t]he evidence provided by applicant need not be conclusive but merely convincing to one skilled in the art.” (MPEP 2164.05 (eighth edition, September 2007); underlining in original.)

Applicants emphasize that in the outstanding Office Action and previous Office Actions the Examiner has only made conclusory statements related to these claims as not meeting the enablement requirement (without a biological deposit). The Examiner has not, as required, provided reasons or evidence why deposits are necessary for the enablement of claims 27-29, 31, 73-75 and 77. Therefore, the Examiner has not made a *prima facie* case of non-enablement.

Furthermore, the Examiner states the “Applicant has failed to demonstrate that the VSV strains designated M1, M2, M3, M4 and M5 are well known and **readily** available to the public **without restriction**.” (Office Action, page 4; emphasis in original.) Applicants respectfully disagree. Not only has the Examiner failed to establish that the recited strains are not well known and not readily available, but Applicants have presented arguments (as below) that provide a *prima facie* showing that the VSV strains recited in claims 27-31 and 73-77 are well known and readily available to the public.

The MPEP states,

[i]n an application where the invention required access to specific biological material, an applicant could show that the biological material is accessible because it is known and readily available to the public. The concepts of “known and readily available” are considered to reflect a level of public accessibility to a necessary component of an invention disclosure that is consistent with an ability to make and use the invention Unless there is a reasonable basis to believe that the biological material will cease to be available during the enforceable life of the patent, current availability would satisfy the requirement **If an applicant has adequately established that a biological material is known and readily available, the Office will accept that showing.**

(MPEP § 2404.01 (eighth edition, September 2007); underlining and bolding added.)

Regarding whether the biological material is readily available, a review of the scientific literature indicates that a variety of researchers have had access to the VSV strains of claims 27-29, 31, 73-75 and 77. Because the strains are generally available to researchers in the field, Applicants respectfully submit that deposit under the terms of the Budapest Treaty is not necessary to meet the enablement requirement.

As evidence of the widespread use of these strains, Applicants previously submitted, as part of an Information Disclosure Statement (IDS) with their Response dated April 7, 2008, copies of the following documents:

- Desforges, *et al.*, Virus Res. (2001) 76(1): 87-102 (Abstract).
- Pasternak, *et al.*, Virology (1988) 166(2): 379-386 (Abstract).
- Marcus *et al.*, J. Gen. Virol. (1980) 47(1): 89-96 (Abstract).
- Ahmed *et al.*, J. Virol. (2003) 77(8): 4646-4657.
- Winship *et al.*, J. Gen. Virol. (1984) 65: 843-847 (Abstract).
- Ferran *et al.*, J. Virol. (1977) 71(1): 371-377.
- Stanners, *et al.*, Cell (1977) 11(2): 273-281 (Abstract).

Desforges *et al.* reports the use of mutant strains T1026 (M1), TP3 (M3) and G31 (M5). Pasternak *et al.* and Marcus *et al.* report the use of mutant strain T1026 R1 (M2). Ahmed *et al.* reports the use of mutant strains T1026R1 (M2), TP2 and TP3 (M3). Winship *et al.* and Ferran *et al.* reports use of mutant strain T1026R1 (M2). Stanners reports use of mutant strain T1026

(M1). Applicants' specification at page 11, lines 23-25, provides a key to the varying nomenclature in the art for these strains.

As further evidence that the VSV strains recited in claims 27-29, 31, 73-75 and 77 are available, many scientific journals require their authors to agree to make biological materials available to the research community. As evidence, Applicants previously provided, in Appendix A of their Response dated April 7, 2008, the following documents:

- 2005 Instructions to Authors, Journal of Virology (Jan. 2005) 79(1): 1-16
- Guide for Authors, Virology (as downloaded July 13, 2005)

The Instructions to Authors wishing to publish in the Journal of Virology states:

[b]y publishing in the journal, the authors agree that any . . . viruses . . . newly described in the article are available from a national collection or will be made available in a timely fashion and at reasonable cost to members of the scientific community for non-commercial purposes.

(p. 2, left-hand column, last full paragraph; bolding in original) The Guide for Authors wishing to publish in Virology states:

[p]ublication of a research article in *Virology* is taken to imply that the authors are prepared to distribute freely to academic researchers for their own use any materials (e.g., viruses, cells, DNA clones, antibodies) used in the published experiments.

(Guide for Authors, Editorial Policies.) These policies indicate these journals require their authors to agree to make biological materials available to the scientific community. These policies and similar ones at other journals as well, provide further evidence that mutant VSV strains as recited in claims 27-29, 31, 73-75 and 77 are publicly available to members of the scientific community. Since the strains are generally available to researchers in the field, Applicants respectfully submit that deposit under the terms of the Budapest Treaty is not necessary to meet the enablement requirement.

Applicants have clearly demonstrated and presented evidence that the viruses recited in claims 27-29, 31, 73-75 and 77 are readily available in the art. In spite of this, during the interview with Applicants' representative on March 5, 2008, the Examiner indicated his concern that the particular viral strains may not continue to be available for the life of the patent.

However, the Examiner has not presented any credible reasons or evidence why they would not continue to be readily available. Without credible reasons or evidence why they would not continue to be readily available, the burden remains with the Examiner to show that claims 27-29, 31, 73-75 and 77 are not enabled.

In summary, Applicants have (i) clearly shown and presented evidence that the VSV strains recited in claims 27-29, 31, 73-75 and 77 are well known and readily available to the public and (ii) the Examiner has not, as required (*e.g.*, see MPEP § 2404.01), provided a reasonable basis to believe that the biological materials are not readily available and will cease to be available during the enforceable life of the patent. The Examiner has not provided any evidence or reasoning to rebut this. Therefore, a biological deposit is not necessary.

Even though Applicants believe that the above completely rebuts the Examiner's rejection, Applicants assert that the VSV strains of claims 27-29, 31, 73-75 and 77 are sufficiently described in the specification and in the art, at the time of the invention, so that one skilled in the art could make and/or use the VSV strains of claims 27-29, 31, 73-75 and 77. For example, Table 11, Figures 14-22, Example 27 and the Sequence Listing of Applicants' specification provide both nucleic acid and amino acid sequence information for viruses that are the subject matter of claims 27-29, 31, 73-75 and 77. Since one skilled in the art at the time of the invention upon review of the present application could have made and/or used the relevant VSV strains, a deposit is not necessary.

(ii) Claims 30 and 76 (related to the M4 strain)

Claims 30 and 76 remain rejected under 35 U.S.C. § 112, first paragraph, for failing to meet the enablement requirements without a biological deposit. Applicants respectfully submit that the claims satisfy the requirements of 35 U.S.C. § 112, first paragraph, and do not require a biological deposit.

The Examiner states,

[t]he deposit of biological organisms is considered by the Examiner to be necessary for the enablement of the current invention (see 37 CFR 1.808(a)).

(July 16, 2008, Office Action, page 4.) Applicants respectfully disagree that a deposit is necessary for the enablement of these claims. Applicants' specification at page 11, lines 23-25, provides a key to the varying nomenclature in the art for the strain M4.

With regards to an enablement rejection, "[t]he evidence provided by applicant need not be conclusive but merely convincing to one skilled in the art." (MPEP 2164.05 (eighth edition, September 2007); underlining in original.)

Applicants emphasize that in the outstanding Office Action and previous Office Actions the Examiner has only made conclusory statements related to these claims as not meeting the enablement requirement (without a biological deposit). The Examiner has not, as required, provided reasons or evidence why deposits are necessary for the enablement of claims 30 and 76. Therefore, the Examiner has not made a *prima facie* case of non-enablement.

During the interview with Applicants' representative on March 5, 2008, the Examiner indicated his concern that the particular viral strains may not continue to be available for the life of the patent. However, the Examiner has not presented any credible reasons or evidence why they would not continue to be readily available. Without credible reasons or evidence why they would not continue to be readily available, the burden remains with the Examiner to show that claims 27-29, 31, 73-75 and 77 are not enabled.

Even though Applicants believe that the above completely rebuts the Examiner's rejection, Applicants assert that the VSV strain of claims 30 and 76 is sufficiently described in the specification and in the art, at the time of the invention, so that one skilled in the art could make and/or use the VSV strains of claims 30 and 76. For example, Table 11, Figures 14-23, Example 27 and the Sequence Listing of Applicants' specification provide both nucleic acid and amino acid sequence information for viruses that are the subject matter of claims 30 and 76.

B. Rejection under 35 U.S.C. § 112, first paragraph, for lack of enablement.

Claims 1, 6-13, 19, 24-37, 64-77 and 79-80 remain rejected under 35 U.S.C. § 112, first paragraph, because:

the specification, while being enabling for methods utilizing attenuated VSV for reducing the viability of hematopoietic tumor cells *in vitro* and the use of attenuated VSV to reduce the viability of tumor cell based xenographs in immunodeficient mice, does not reasonably provide enablement for the utilization [of] attenuated VSV for the reduction of viability of all types of hemapoietic tumor cells to reduce the viability of a tumor cell in an immunocompetent animal.

(July 16, 2008, Office Action, page 5.) Applicants respectfully disagree.

The purpose of the enablement requirement is to ensure that the specification describes the invention in such terms that one skilled in the art can make and use the invention commensurate with the scope of the claims. (*E.g.*, see MPEP § 2164 (eighth edition, September 2007).)

The Patent and Trademark Office (PTO) bears the initial burden of providing reasons for doubting the objective truth of the statements made by applicants as to the scope of enablement. Only when the PTO meets this burden, does the burden shift to applicants to provide suitable evidence indicating that the specification is enabling in a manner commensurate in scope with the protection sought by the claims. *In re Marzocchi*, 439 F.2d 220, 223, 169 USPQ 367, 369 (CCPA 1971). **The Examiner has not provided reasons for doubting that one skilled in the art could reduce the viability of hematopoietic tumor cells, in vitro or in vivo, using the claimed methods.**

What is pertinent to meeting the enablement requirement is whether one skilled in the art can make and use the invention commensurate with the scope of the claims. In other words, with regards to the present claims, **can one skilled in the art use the claimed methods to reduce the viability of a hematopoietic tumor cell with administration of a VSV?** Applicants have clearly demonstrated this, as discussed below, regardless of whether the administration is *in vitro* or *in vivo*. The Examiner has provided no evidence to the contrary.

The Examiner's rejection is deficient and legally insufficient in that, *inter alia*, (A) the rejection focuses on whether Applicants' specification has enabled claims directed to treating cancer and/or efficacy in a human⁶ and (B) Applicants have demonstrated that upon review of

⁶ For clarity, Applicants believe that, if presented, similar claims to treating hematopoietic tumor cells are enabled by the present application.

Applicants' specification, one skilled in the art, at the time of the invention, would have been able to practice the claimed invention without undue experimentation.

(i) Improper focus on "efficacy" and "therapeutic effect".

Even though the practice of the claimed invention may result in a therapeutic benefit, the subject matter of the claims relates to "reducing the viability of a tumor cell". The present claims do not recite or require any "efficacy" or "therapeutic effect". However, throughout prosecution, the Examiner has inappropriately read the requirement for efficacy and/or therapeutic effect into the claims. For example, the Examiner states,

contrary to applicant's assertion [that there is no limitation to treat cancer], the reduction in the viability of a tumor cell in the context of a living being . . . constitutes a therapeutic response Consequently, clinical response is pertinent with regard to the enablement of the instant claims.

(July 16, 2008, Office Action, page 6.) The Examiner also states,

the specification does not provide any basis for correlating the *in vitro* results with beneficial effects that could reasonably be expected when said viruses are administered *in vivo* to "treat" hematopoietic tumor cells, although *in vivo* use is clearly encompassed by the claims. [sic] Lacking either direct evidence for *in vivo* benefit, or a reasonable basis for correlating the *in vitro* and xenograft data as exemplified in the instant specification with *in vivo* benefit. Hence, the specification cannot be said to teach how to use the claimed viruses as pharmaceuticals without undue experimentation.

(November 5, 2007, Office Action, page 20, underlining added.) On the contrary, whether or not a clinical response can be shown or predicted is not pertinent for meeting the enablement requirement with regards to the claimed invention, since the claims do not recite any limitations directly related to a therapeutic reduction of tumor cell viability or clinical response.⁶ The Examiner seems to be confusing the differences between therapeutic applications and reducing the viability of a tumor cell. What is pertinent is whether one skilled in the art can make and use the invention, *i.e.* to reduce the viability of a hematopoietic tumor cell with administration of a vesicular stomatitis virus (VSV), commensurate with the claims.

In the initial paragraph of the enablement rejection (July 16, 2008, Office Action, page 5.), the Examiner refers to reducing the viability of a tumor cell, but then focuses on arguments and documents allegedly showing that certain models and/or experiments do not correlate with efficacy or treatment in humans. For example, the Examiner states,

Gura . . . teach that xenographs are not good models for determining the efficacy of a treatment modality Gura illustrates the lack of correlation between efficacy in xenograft model systems and in vivo efficacy in humans.

(July 16, 2008, Office Action, page 12, underlining added.) However, the Examiner has not expressed specific reasons why one skilled in the art, upon review of Applicants' specification would not have expected the claimed methods to result in the reduction of the viability of a tumor cell in vivo or in an immunocompetent animal.

To further support this position, Applicants referred the Examiner to *Ex parte Saito and Zhao* (Appeal No. 2005-1442 before the Board of Patent Appeals and Interferences (BPAI), not binding precedent of the Board) and *Ex parte Boutin* (Appeal No. 2006-1879 before the BPAI, not binding precedent of the Board), which both stand for the proposition that unless the claims explicitly refer to a therapeutic benefit, typically the Examiner should not determine if the claims are enabled for an unclaimed therapeutic benefit. In *Ex parte Saito* and *Zhao* the Board stated,

the examiner may be correct that achieving clinically useful gene therapy using the claimed method would require undue experimentation, but the claims are not nonenabled merely for encompassing that difficult-to-achieve outcome.

(*Ex parte Saito* and *Zhao*, page 7.) In *Ex parte Boutin* the Board stated,

when the claims are not directed to a method that achieves a therapeutically useful result, achieving such a result is not required for the claims to be enabled Thus, while the claims read on gene therapy methods, they do not require producing a clinically effective therapeutic response.

(*Ex parte Boutin*, page 6.) The *Ex parte Boutin* decision also states,

[t]his appeal involves claims to a method of transferring nucleic acids into cells, which the examiner has rejected as nonenabled Because we conclude that enabling the claimed method does not require providing therapeutically effective gene therapy, we reverse.

(*Ex parte Boutin*, page 1.)

The claims in both *Ex parte Saito and Zhao* and *Ex parte Boutin* required expression of a gene but did not require a therapeutic result. Both of these decisions stand for the proposition that to satisfy the enablement requirement, all that was required is the expression of the transgene and not a therapeutic benefit. This is similar to the present application in that the claims require reduction of the viability of a hematopoietic tumor cell, but not a therapeutic benefit. The

Examiner has provided no evidence that one skilled in the art would not be able to reduce the viability of a hematopoietic tumor cell, *in vitro* or *in vivo*. The Examiner has not expressed specific reasons why one skilled in the art, upon review of Applicants' specification would not have expected the claimed methods to result in the reduction of the viability of a tumor cell. At most, some of the references cited by the Examiner (discussed in more detail below) may suggest that some drug candidates with positive results in a xenograft model are not later approved or used as drugs in people. However, a cancer drug candidate may fail to become an approved drug for various reasons including (i) the reduction in tumor cell viability may not meet a justifiable or predetermined level, (ii) the general toxicity may be too great, (iii) the therapeutic benefit does not justify the cost and/or (iv) the therapeutic results are not equivalent to or better than a standard of care. Therefore, cancer drug candidates can fail to be FDA approved treatments even though they are shown to reduce the viability of tumor cells in a patient. None of the references cited by the Examiner demonstrate or suggest that, upon review of the present specification, one skilled in the art would not have been able to reduce the viability of a hematopoietic tumor cell *in vitro* or *in vivo* using the claimed methods.

The Examiner responded to Applicants' discussion of *Ex parte Saito and Zhao* and *Ex parte Boutin* by stating that, "contrary to applicant's assertion, the reduction in the viability of a tumor cell in the context of a living being . . . constitutes a therapeutic response." (July 16, 2008, Office Action, page 6.) In the following paragraph of the Office Action, the Examiner attempts to distinguish the present situation from these two board decisions in that they "are not germane to the instant application as . . . the instant claims refer to a therapeutic response." (July 16, 2008, Office Action, page 6.) Applicants agree that in some cases practice of the methods claimed herein may result in a therapeutic result, but do not agree that the claims require a therapeutic response.

In addition to the two BPAI decisions referred to above, Applicants referred the Examiner to *Ex parte Ayishi* (Appeal No. 2006-1608 before the BPAI, not binding precedent of the Board). This case is similar to the present case in that the claims do not specifically recite or require a therapeutic effect, but recite a method that may encompass methods capable of achieving a clinically effective therapeutic response. In *Ex parte Ayisi* the Board stated,

The invention that must be enabled to satisfy § 112 is the invention defined by the claims. See CFMT, Inc. v. Yieldup Int'l Corp., 349 F.3d 1333, 1338, 68 USPQ2d 1940, 1944 (Fed. Cir. 2003) (Title 35 does not require that a patent disclosure enable one of ordinary skill in the art to make and use a perfected, commercially viable embodiment absent a claim limitation to that effect.). **Thus, when the claims are not limited to a method that achieves therapeutic or clinical efficacy, such efficacy is not required for the claims to be enabled.**

Here, the claims are directed to a method comprising [] contacting a virus-infected cell with an extract from Ocimum gratissimum in an amount effective to inhibit cytopathic effects of the virus in the cell (claim 31). Thus, while it is fair to say that the claims encompass a method that achieves a clinically effective therapeutic response, they do not require it. Cf. In re Cortright, 165 F.3d 1353, 49 USPQ2d 1464 (Fed. Cir. 1999) (claims to a method of treating scalp baldness could be enabled even if the method did not produce a full head of hair).

We conclude that the potential problems identified by the examiner may indeed complicate treatment of a HIV in a patient, but such problems need not be overcome in order to contact[] a virus-infected cell with an extract from Ocimum gratissimum in an amount effective to inhibit cytopathic effects of the virus in the cell - all that is required by the claims. Thus, the examiner has not adequately explained why practicing the claimed method would have required undue experimentation.

Moreover, a claim may encompass inoperative embodiments and still meet the enablement requirement of 35 U.S.C. § 112, first paragraph. See Atlas Powder Co. v. E.I. Du Pont De Nemours & Co., 750 F.2d 1569, 1576, 224 USPQ 409, 413 (Fed. Cir. 1984), In re Angstadt, 537 F.2d 498, 504, 190 USPQ 214, 218 (CCPA 1976), In re Cook, 439 F.2d 730, 732, 169 USPQ 298, 300 (CCPA 1971). And the stage at which an invention in this field become useful is well before it is ready to be administered to humans. In re Brana, 51 F.3d 1560, 1568, 34 USPQ2d 1436, 1442 (Fed. Cir. 1995). (While the Brana court referred to usefulness, the rejection on appeal was for nonenablement. See id. at 1564, 34 USPQ2d at 1439.)

Therefore, as the examiner has failed to set forth a prima facie case of unpatentability under 35 U.S.C. § 112, first paragraph, we are compelled to reverse the rejection.

(*Ex parte Ayisi*, pages 5-7, underlining in original; quotation remarks removed, bolding added.)

Applicants assert that the enablement issues of *Ex parte Ayisi* are relevant, at least in part, to those in the present application. In the case of *Ex parte Ayisi*, the claims refer to a method comprising contacting a virus-infected cell with an extract from Ocimum gratissimum in an amount effective to inhibit cytopathic effects of the virus in the cell, whereas the present claims

refer to methods of reducing the viability of a tumor cell, comprising administering to the tumor cell a vesicular stomatitis virus.

Therefore, the Examiner has improperly focused on enabling therapeutic treatment and has not provided specific reasons why one skilled in the art would not have been enabled to reduce the viability of a hematopoietic tumor cell commensurate with the claims.

(ii) Claims Are Enabled and Xenograft Models Are Predictive for Claimed Invention

Several of the documents cited by the Examiner actually conclude that in the case of cytotoxic agents, xenograft models are predictive of clinical outcome. For example, Peterson *et al.* (Eur. J. Cancer 2004 40:837-844) states:

from the perspective of drug sensitivity, at least to the conventional cytotoxic agents that comprise most of our current experience, the subcutaneous models appear relatively predictive.

(page 838, first column.) Along the same lines, Kelland (Eur. J. Cancer. 2004, 40(6):827-836) states

one may reasonably conclude that, at least for cytotoxic cancer drugs, the human tumour xenograft model, is a good predictor of clinical activity.

(page 83, first column.) *Inter alia*, VSV can be thought of as cytotoxic agent for hematopoietic tumor cells. Therefore, these documents cited by the Examiner support a conclusion that the claimed invention is enabled.

Applicants' previous Reply of November 24, 2004 discussed McCormick (U.S. Patent No. 5,677,178) and Pecora (Pecora *et al.* J. of Clin. Oncol. 20(9):2251-2266 (2002)). *Inter alia*, McCormick and Pecora are presented as showing that viral therapy results, using *in vitro* assays and *in vivo* xenograft models, reasonably correlate with clinical results. The only experimental results contained in McCormick are the results of *in vitro* testing (Patent No. 5,677,178, column 18, line 42 to column 20, line 23; and Figures 2A-3C). Nevertheless, McCormick bases his teaching of human therapy on those *in vitro* results (Patent No. 5,677,178, column 16, line 45 to column 18, line 25). The Examiner attempted to devalue the importance of McCormick by noting that "it is well settled that whether similar claims have been allowed to others is immaterial." (May 26, 2004 Office Action, page 8). That statement of the law is fair enough, but

it missed the point that Applicants do not cite McCormick for its status as an issued patent, but rather for the fact that it illustrates that McCormick, like so many others, considered *in vitro* results to support his teaching of human therapy. T

Additionally, the McCormick patent relates to ablating neoplastic cells using an E1b deleted adenoviral vector. Onyx-015 is an E1b deleted adenoviral vector that was shown to have efficacy in human clinical trials. (*E.g.*, see Nemunaitis *et al.* Journal of Clinical Oncology, 19(2):289-298 (2001).) Pecora describes positive Phase I data using a replication competent strain of Newcastle Disease Virus. Both Pecora and McCormick demonstrate that the results from *in vitro* and *in vivo* models, described in each article, have a reasonable correlation with clinical results. The same or similar models have been utilized in Applicants' specification and in the articles discussed below.

However, the Examiner dismissed the value of the Pecora and McCormick documents saying, "since neither McCormick nor Pecora utilize VSV to treat hematopoietic cancers, they cannot be relied upon to 'demonstrate' that *in vitro* data correlates with *in vivo* efficacy."⁷ (February 7, 2007, Office Action, page 10.) But, of course the arguments of the rejection are not specific to VSV either, but rather relate to the alleged difficulty of correlating *in vitro* results to *in vivo* efficacy in the cancer field generally. Thus, the citations to Pecora and to McCormick are properly cited to refute the rejection. Applicants respectfully assert that these two documents are more relevant than the articles relied on by the Examiner in an attempt to support an enablement rejection. McCormick and Pecora use assays and models to test viral therapy and support the position that these models have a reasonable correlation with clinical results. Applicants could not find any passages in the articles cited by the Examiner that even discuss assays or models with regards to viral therapy, let alone related to the utilization of VSV. In fact, most if not all of the articles relied on by the Examiner relate to small molecule drug evaluations.

⁷ Following the Examiner's logic, the references referred to by the Examiner cannot be relied upon to allegedly "demonstrate" that *in vitro* or *in vivo* data does not correlate with clinical efficacy.

Applicants previously provided the following journal articles related to reducing the viability of a hematopoietic tumor cell(s) comprising administering a VSV virus to the tumor cell(s).

- (i) Lichty *et al.* (Human Gene Therapy, 15:821-831 (2004))
- (ii) Césaire *et al.* (Oncogene, 25:349-358 (2006))
- (iii) Porosnicu *et al.* (Cancer Research 63:8366-8376 (2003))
- (iv) Balachandran and Barber (IUBMB Life 50:135-138 (2000))
- (v) Stojdl *et al.* 2003 (Cancer Cell, 4:263-275 (2003))
- (vi) Stojdl *et al.* 2000 (Nature Medicine, 6(7):821-825 (2000))

These articles were published subsequent to Applicants' priority filing (U.S. Patent Application No. 60/287,590, filed September 17, 1999). These articles provide evidence that at the time of Applicants' priority filing, one skilled in the art using the teachings in Applicants' specification would have been enabled to use the presently claimed methods for reducing the viability of a tumor cell(s) by administering a vesicular stomatitis virus to the tumor cell(s).

In particular, Lichty *et al.* shows, *inter alia*, the following: (1) Four distinct VSV virus strains were able to kill at least 11 of 12 cell lines in a panel of human leukemic cell lines (*e.g.*, see page 821, Overview Summary paragraph and Table 1); (2) Two VSV viruses were successfully used to purge leukemia cells from mixed cultures containing peripheral blood stem cells (see, *e.g.*, paragraph bridging page 827-828 and Table 3); and (3) VSV viruses kill myeloma cells from primary patient samples (see, *e.g.*, paragraph bridging column 1 to column 2 on page 828 and Figure 3). Therefore, Lichty *et al.* shows both (i) a reduction of viability in most, if not all, hematopoietic tumor cell types by administering a VSV virus and (ii) a reduction of viability of hematopoietic tumor cells from human patients.⁸ This article also shows the selective reduction in viability of hematopoietic tumor cells (leukemic cells) in a population comprised of peripheral blood stem cells. Lichty *et al.* concludes the article by stating:

⁸ Lichty *et al.* also states, "[w]e have been struck by the extreme resistance of normal bone marrow progenitors to VSV infection . . . it is clear that bone marrow stem cells and normal peripheral blood lymphocytes have active antiviral programs that rapidly blunt virus infections."

[t]aken as a whole these observations and the results reported in this study point to the utility of VSV as a leukemolytic agent for the *in vivo* and *ex vivo* treatment of hematologic malignancy.

The results described in Lichty *et al.* provide, at minimum, a reasonable correlation that Applicants' claimed methods, related to reducing the viability of a tumor cell, are applicable *in vitro*, *ex vivo*, or *in vivo* (e.g., in a human), especially in light of the fact that Lichty *et al.* describes results using human patient samples. Therefore, Lichty *et al.* demonstrates that Applicants' claimed invention is enabled.

Césaire *et al.* tested four adult T-cell leukemia samples from four human patients. All four samples "underwent rapid oncolysis in a time dependent manner" upon administration of a VSV virus. (E.g., see abstract and Table 1.) Additionally, a reduced viability was observed upon administration of a VSV virus to 2 of 2 HTLV-1 transformed T-cell lines and 2 of 2 B-cell chronic lymphocytic leukemia (B-CLL) cell lines.⁹ (E.g., see page 351, first column, last paragraph and page 353, first column, respectively.) "[N]onleukemic cells from patients with HAM/TSP [HTLV-1-associated myelopathy/tropical spastic paraparesis] were resistant to VSV infection." (Page 355, second column.) Similar to Lichty *et al.*, Césaire *et al.* also describes results using human patient samples. Therefore, Césaire *et al.* demonstrates that Applicants' claimed invention is enabled.

Porosnicu *et al.* shows that infection of K562 cells (human leukemia cell line) and EL4 cells (T-cell lymphoma) with 4 different VSV viruses resulted in >75% cell death within 24 hours. (E.g., see page 8370, second column, first full paragraph and Figure 4). These four viruses were also tested *in vivo* in an immunocompetent mice model using A20 cells (B-cell lymphoma cells). The Examiner has referred to Bibby as teaching that "in the interest of finding more clinically relevant models, orthotopic models have been developed." (February 7, 2007, Office Action, page 8.) In fact, the immunocompetent mice model using A20 cells is an

⁹ Césaire *et al.* also tested three B-CLL and one T-CLL from patients, *ex vivo*. According to Césaire *et al.* these samples were not permissive to VSV replication. (E.g., see page 353, first column and Table 1.) However, Césaire *et al.* states, "[t]his discrepancy in VSV oncolysis may be due to the fact that CLL cells do not proliferate but remain in G₀ *ex vivo* (Meinhardt *et al.*, 1999; Caligaris-Cappio, 2003)." (Page 357, second column.)

orthotopic model, in which Porosnicu *et al.* demonstrated a significant reduction of *in vivo* tumor growth for each of the four VSV viruses as compared to the control groups. (*E.g.*, see page 8371, column1 and Figure 5.)

Balachandran and Barber show that 4 of 4 hematological malignancies tested had their viability reduced by administration of a VSV virus. Balachandran and Barber state:

[t]o further examine the ability of VSV to induce cell death in other transformed human cell lines, including those derived from . . . various cells derived from hematological malignancies (HL 60, K562, Jurkat, BC-1), we infected those cells with VSV as described in Experimental Procedures. We observed that VSV efficiently replicated and induced cytolysis of every established cell line tested.

(Page 136, paragraph bridging columns 1 and 2.)

Stojdl *et al.* 2003 also screened a panel of leukemia cell lines and found that 4/6 cell lines tested were deemed highly sensitive to wild-type VSV infection, *e.g.*, see Table 2A. This document is silent as to if the other two cell lines were sensitive, but not highly sensitive, to VSV infection.

Stojdl *et al.* 2000 shows that “acute myelogenous leukemia (AML) cell lines OCI/AML3, OCI/AML4 and OCI/AML5 were very susceptible to VSV infection”. (*E.g.*, see page 822, column 2.) Additionally, this article shows that VSV had selective oncolytic properties in a co-culture of leukemic OCI/AML3 cells mixed with normal human bone marrow cells (at a ratio of 1:9). (*E.g.*, see page 22, column 2, first sentence.)

The articles discussed in this section provide clear evidence that the viability of hematopoietic tumor cells, in general, are reduced upon administration of a vesicular stomatitis virus. Additionally, these articles demonstrate that the claims, as presented herein, are enabled.

(iii) Articles Cited by the Examiner to Allegedly Support Enablement Rejection

The following articles are being cited by the Examiner to allegedly support the enablement rejection. Applicants discuss why each of them does not support the enablement rejection of the claimed invention. As a general point, many of these articles actually provide support that (i) the claims are enabled and (ii) even that one skilled in the art would conclude that

the *in vitro* and/or *in vivo* results in Applicants' specification would reasonably correlate with results in other animals, such as humans.

Kelland (Eur. J. Cancer. 2004, 40(6):827-836)

The Examiner's summaries of Kelland do not directly relate to or compare statements in Kelland to Applicants' claimed invention. Therefore, Applicants are not able to specifically address Kelland with regards to the current enablement rejection of the present claims.

On the other hand, Kelland, which is cited by the Examiner, provides various rationale which supports the premise that the *in vitro* and *in vivo* results in Applicants' specification and in the articles discussed above are predictive of results in a human.

Kelland provides analyses that indicate whether or not human tumor xenograft models might be predictive of clinical results. Kelland summarizes these analyses stating:

[o]verall, taking all of the above into consideration, one may reasonably conclude that, at least for cytotoxic cancer drugs, the human tumour xenograft model, is a good predictor of clinical activity.

(Kelland, page 831, first column; underlining added). In fact when referring to Kelland, the Examiner stated that "the successful use of such models in cytotoxic drug development is conclusive." (July 16, 2008, Office Action, page 13.) VSV viruses can reduce the viability of a tumor cell, *inter alia*, via cytotoxic effects. Therefore, successful results are a "good predictor of clinical activity" and demonstrate that the claimed subject matter meets the requirements for enablement. Kelland goes on to state that,

[i]n careful mechanism-based studies, combined with sound pharmacological principles (as described above), then, in my view, the xenograft model remains of great value, both for assisting in the selection of leads for clinical evaluation and for guiding clinical studies.

(Kelland, page 833, second column.) Although not wishing to be bound by theory, Applicants' specification provides possible mechanisms related to the claimed methods. For example, differential susceptibility of a tumor cell to a VSV virus can be more pronounced in the presence of interferon. Also, differential susceptibility of a tumor cell to a VSV virus can be related to, *inter alia*, the PKR status of a tumor cell. Some, if not all, of the *in vitro* and *in vivo* studies described in the present application and in the articles described above may be considered as

mechanism-based studies and, according to Kelland, are therefore of assistance in guiding clinical studies.

The Examiner admits that “Kelland discloses that the xenograft model is an effective screen for candidates for Phase I clinical trials”. (July 16, 2008, Office Action, page 7.) If a drug candidate is approved for a Phase I clinical trial, then obviously those skilled in the art expect a correlation between the preclinical results and the expected clinical results. Therefore, based on the Examiner’s summary of Kelland, those skilled in the art consider the xenograft model to reasonably correlate with clinical outcomes. Otherwise, Kelland would not conclude that the xenograft model is an effective screen for candidates for Phase I clinical trials.

In summary, Kelland clearly supports the position that one skilled in the art would conclude that the models and related results, described in Applicants’ specification and in the articles cited above, reasonably correlate with an expected similar result in other animals, such as humans.

Wang *et al.* (Exp. Opin. Biol. Ther. 2001, 1(2):277-290)

Although the Examiner continues to cite Wang *et al.*, Applicants are unclear as to the relevance of this article with regards to the presently claimed invention. Wang *et al.* relates to T-cell-directed cancer vaccines, whereas the claimed invention relates to methods of reducing the viability of a hematopoietic tumor cell(s) comprising administering to the hematopoietic tumor cell(s) a VSV virus.¹⁰ Therefore, Applicants do not understand the relevance of the sections of Wang *et al.*, referred to by the Examiner, to the subject matter of the claims as presented herein. Additionally, the Examiner has not described how the disclosure of Wang *et al.* relates to the results in Applicants’ specification or to the presently claimed invention.

Gura (Science, 1997, 278:1041-42)

Gura discusses historical results in a general and broad sense. Applicants’ presently claimed methods relate to, *inter alia*, reducing the viability of a hematopoietic tumor cell(s),

¹⁰ Although, Applicants acknowledge that *in vivo* the immune system may contribute to reducing the viability of a tumor cell.

comprising administering to the tumor cell(s) a VSV virus. Gura does not speak to the predictive value of models related to evaluating the administration of a virus to a tumor cell. Therefore, Gura bears little, if any, relevance to the presently claimed invention or related *in vitro* or *in vivo* experiments.

Voskoglou-Nomikos *et al.* (Clin. Cancer Res. 2003 9:4227-4239)

Again, the Examiner has not related the disclosure in Voskoglou-Nomikos *et al.* to the claimed invention. Referring to Voskoglou-Nomikos *et al.*, the Examiner states that “xenograft models were only predictive for non-small cell lung [cancer] and ovarian cancers, but not for breast and colon cancers”. (February 7, 2007, Office Action, page 6.) Voskoglou-Nomikos *et al.* appears to be silent with regards to predictability of the xenograft models related to the administration of a VSV virus to a hematopoietic tumor cell. Also, Voskoglou-Nomikos *et al.* suggests predictability can vary with specific models. Therefore, Voskoglou-Nomikos *et al.* provides no evidence as to whether *in vitro* or *in vivo* experiments related to administering a VSV virus to a hematopoietic tumor cell(s) reasonably correlate to expected results in other animals, such as humans.

Saijo *et al.* (Cancer Science 2004, 95(10):772-776)

The Examiner indicated that,

Saijo *et al.* discloses that while numerous phase III trials have been conducted upon the basis of promising preclinical data such as that disclosed in the instant application, few have yielded strongly positive results, and the majority of results have been negative (e.g., abstract).

(July 16, 2008, Office Action, pages 6-7; underlining added.) Most negative phase III results do not prove statistical inactivity. Instead these “negative” results can indicate that a certain degree of activity has not been met in the clinic for the sample size tested. Even Saijo admits finding active drugs in the clinic are often hampered by having extremely small sample sizes (e.g., see page 774; second column). Economic factors come into play when determining sample sizes. Of course, typically drugs that have “strongly positive” results will allow for a phase III trial to be successful with a small sample size. Applicants note, the present claims do not require “strongly positive” results.

Applicants note that Saijo focuses on whether the results of preclinical studies for molecular-target-based drugs correlate with results seen in clinical trials. Applicants do not consider the disclosure of Saijo to have particular relevance to methods of reducing the viability of a hematopoietic tumor cell(s) comprising administering to the tumor cell(s) a VSV virus. As noted herein, Voskoglou-Nomikos *et al.* suggests predictability can vary with specific models.

Schuh (Toxicologic Pathology 2004, 32(Suppl. 1):53-66)

Schuh states “[c]ommon reliance on survival and tumor burden data in a single mouse model often skews expectations towards high remission and cure rates; seldom duplicated in clinical trials.” (Abstract; underlining added.) As Applicants show herein, administration of VSV virus in several mouse models using hematopoietic tumor cell types resulted in reducing the viability of the hematopoietic tumor cells. Therefore, one skilled in the art would expect these results to reasonably correlate with results expected in a human.

Bibby (Eur. J. Cancer 2004 40(6):852-857)

The Examiner refers to Bibby as teaching that “in the interest of finding more clinically relevant models, orthotopic models have been developed”. (July 16, 2008, Office Action, page 8.) Bibby discusses whether to use orthotopic models, for example, as opposed to some xenograft models. However, Bibby does not teach that results in xenograft models do not reasonably correlate with clinical results in another animal or a human. In fact with regards to a hematopoietic cell(s), Bibby states:

In the past, murine tumour systems were used for drug screening with mouse leukaemias being utilised as prescreens [1]. These grew very rapidly, had a high growth fraction and proved to be sensitive to a number of agents that were subsequently shown to have more activity against leukaemias and lymphomas than against solid carcinomas and sarcomas and to be toxic to the bone marrow [2]. As a result of these early screens, there is a general misconception that tumours in rodents are sensitive to drug therapy and are easy to cure. In reality this is untrue and back in 1987 Corbett and colleagues [3] pointed out that most of the agents that had entered the clinic at that time had poor or no activity against the majority of transplantable solid tumours in mice. Modest activity is often seen, but this is usually at the expense of host toxicity [4].

(page 852.) Bibby clearly stands for the proposition that with regards to leukemias “there is a general misconception that tumours in rodents are sensitive to drug therapy and are easy to cure” and that “[m]odest activity is often seen.” Therefore, one skilled in the art would conclude that methods resulting in the reduction of the viability of a hematopoietic tumor cell(s) in a rodent would reasonably correlate with results obtained in a human.

Peterson *et al.* (Eur. J. Cancer 2004 40:837-844)

Peterson *et al.* discusses methods that might improve the predictability of results in a xenograft model. Peterson *et al.* does not teach that results in xenograft models do not reasonably correlate with clinical results in another animal or a human. Therefore, Applicants are unclear as to the relevance of Peterson to the subject matter of the claims presented herein.

The Examiner states “Peterson *et al.* teaches numerous agents have show [sic] exciting activity in preclinical models and yet have had minimal activity clinically”. (July 16, 2007, Office Action, page 16.) Therefore, Peterson is not saying they had no activity.

(iv) The Claimed Invention Is Enabled With Regards To The Genus Of Hematopoietic Tumor Cells

Applicants’ previous Replies have shown that numerous tumor cell lines, representing at least 9 different hematopoietic tumor types, are susceptible to VSV infection. This represents a wide range of tumor cell types and therefore demonstrates enablement commensurate with the scope of the present claims.

The specification teaches that “VSV has a broad host range and is capable of infecting most types of human cells, whereas other viruses are more limited in regard to the types of cells they may effect”. (Specification, page 6, lines 27-29). In view of the broad host range of VSV, the person of ordinary skill in the art would not require undue experimentation to practice the claimed invention.

There are ample data supporting the claim that hematopoietic cancers are, in general, appropriate targets for therapy with VSV (see Table A below which summarizes data within the specification). Six different classes of hematopoietic cancer cell lines were tested in many

different experiments and found to be susceptible to VSV including megakaryocytic leukemia, lymphoid leukemia, acute myelogenous leukemia, chronic myelogenous leukemia, T cell leukemia and myeloma (Table A below). Further supporting evidence within the application is provided by the breadth of tumor cell types that are shown to be sensitive to VSV-mediated killing. The data in Table 1 of the application show that a broad panel of tumor cell types is sensitive to killing by VSV. Together, the data within the specification demonstrates that a wide variety of tumor cell types, and specifically hematopoietic cancers, are appropriate targets for therapy with VSV.

Table A. Diverse Hematopoietic Cancers Were Found to be Sensitive to VSV as Indicated in the Specification.

Cell Line Sensitive to VSV	Type of hematopoietic cancer	Data in Specification
MO7E	Megakaryocytic Leukemia	(1) Table 1 (2) Example 2: "Two leukemia cell lines MO7E and L1210; Table 1) were killed following an overnight infection and produced large amounts of virus." (page 30 lines 11-12); (3) Specification (page 19, lines 29-31): "In contrast two leukemia cell lines (MO7E and L1210) were also tested and found to be susceptible to VSV infection as evidenced by cytopathic effect, virus growth and loss of cell viability."
L1210	Lymphoid Leukemia	As noted above.
AML5	Acute Myelogenous Leukemia	(1) Specification (page 29, lines 12-14): "Other cell lines, including a lung carcinoma cell line (LC80) and a leukemia cell line, AML5 (acute myelogenous leukemia 5) cells were also found

		to be effectively killed by VSV.”
AML3	Acute Myelogenous Leukemia	(1) Example 14 AML3 cells die by apoptosis following infection with VSV. See entire example. (2) Example 15 Mutant VSV strains infect and kill AML cells. See entire example. (3) Example 26 Selective killing of AML cells co-cultured with normal bone marrow; Also see Table 10.
Primary acute myelogenous leukemia	Acute Myelogenous Leukemia	(4) Specification (page 30, lines 25-26): “These results demonstrate VSV is able to preferentially kill primary leukemia blast cells while sparing normal blood cells.”
K-562	Chronic Myelogenous Leukemia	Example 19 and Table 8. “This example shows that VSV is able to infect and kill a diverse set of leukemia cell types.”
MOLT-4	T Cell Leukemia	Example 19 and Table 8
SR	Myeloma	Example 19 and Table 8
H929	Myeloma	Example 19 and Table 8

As discussed above, additional research performed after the filing of the application demonstrates that at least six additional hematopoietic cancer cell lines (LY-8 B-cell lymphoma; LY-18 B-cell lymphoma; Jurkat acute lymphoblastic leukemia; OCI-My10 myeloma; OCI/AML1 acute myeloid leukemia; H191 acute myeloid leukemia) were all sensitive to killing by VSV (Lichty *et al.* 2004, Human Gene Therapy 15:821-31).

Furthermore, the specification teaches characteristics for which one skilled in the art can, without undue experimentation, screen a particular hematopoietic tumor cell type to confirm its sensitivity to VSV, *e.g.*, screen for reduced or no activity of (i) PKR, (ii) PML (iii) STAT1, and/or (iv) interferon regulatory factor (IRF-1) and/or interferon (see *e.g.*, page, 4 lines 8-22;

page 11, lines 21-27; page 12, line 7 to page 16, line 22; and Example 1).¹¹ For example, “results obtained for Table 1 (in the specification) demonstrate that a screening strategy for determining the types of tumours which are susceptible to killing by VSV may be employed”. (Specification, page 29, lines 29-31.) The level of testing taught in the application and known in the art is well within the capabilities of one skilled in the art, at the time of filing, and would not require undue experimentation.

While no model is perfect, Winograd concluded that there is “a good predictability of a panel of human tumor lines for clinically effective drugs” and that “the application of human tumor xenografts in anticancer drug development is warranted.” (B. Winograd, *et al.*, “Human tumor xenografts in the nude mouse and their value as test models in anticancer drug development (review)” *In Vivo* (1987) 1: 1-14, Abstract on p. 1). In the oncology reference Cancer Principles & Practice of Oncology (2001; 6th edition; editors: DeVita VT, Jr, Hellman S, and Rosenberg SA), Chu *et al.* (Section 19.2, para. bridging pp. 352-353) indicate that as part of the standard NCI development scheme for new cancer drugs, the human tumor cell line most sensitive to an active candidate *in vitro* is selected for testing *in vivo* as a xenograft in a subcutaneous implant site in a nude mouse. The U.S. FDA typically accepts positive tumor xenograft results as a sufficient level of preclinical activity when approving a clinical trial for an investigational new drug (IND).

It is, of course, important not to confuse “the requirements under the law for obtaining a patent with the requirements for obtaining government approval to market a particular drug for human consumption.” *In re Brana*, 51 F.3d 1560, 1567, 34 USPQ2d 1436, ____ (Fed. Cir. 1995), citing, *Scott v. Finney*, 34 F.3d 1058, 1063, 32 USPQ2d 1115, 1120 (Fed. Cir. 1994).

In maintaining the rejection the Examiner has cited several articles, which together demonstrate nothing more than the unsurprising observation that the *in vitro* environment cannot duplicate the *in vivo* environment exactly. Nevertheless, it is undeniable that *in vitro* experiments continue to be performed and relied upon to identify treatments for *in vivo* use. If

¹¹ For clarity, with the exception of claims 19 and 71, Applicants’ invention, as claimed herein, is not limited to reducing the viability of tumor cells with these characteristics.

the rejection was correct that “clinical correlations are generally lacking” (December 30, 2002, Office Action, page 6.), *in vitro* experiments would not be as widely used as they are.

Additionally, Applicants refer to the prosecution of U.S. Patent Application No. 11/685,483 (‘483 application(abandoned)), which claims priority to the present application and was examined by the same Examiner as the instant application. Claim 1 of this related application recited:

[a] method of reducing the viability of a tumor cell, comprising administering to the tumor cell a vesicular stomatitis virus, wherein said tumor cell is a carcinoma, wherein the virus is contained in a cell infected with the virus, and wherein the administering comprises administering the virus-infected cell.

The tumor cell in this claim was a carcinoma, whereas the tumor cell in claim 1 of the present application is a hematopoietic tumor cell. In an obviousness rejection of, *inter alia*, the claim 1 of the ‘483 application, the Examiner states that “the use of VSV as a cancer treatment is well known in the art yielding predictable results”. (April 10, 2008, Office Action, page 21, for U.S. Patent Application No. 11/685,483.)

Something well known in the art yielding predictable results is clearly enabled. If the use of VSV as a cancer treatment is well known in the art yielding predictable results, then it follows that, at the time of the claimed invention, one skilled in the art upon review of Applicants’ specification would consider that a method, as claimed herein, of reducing the viability of a tumor cell comprising administering to the tumor cell a vesicular stomatitis virus infected cell is clearly enabled. Therefore, based on the specification, Applicants’ remarks and/or as confirmed by the Examiners’ own admission, claims 1, 6-13, 19, 24-37, 64-77 and 79-80 are enabled.

(v) Summary

In summary, no reasons or evidence have been presented that show or suggest that one skilled in the art cannot reduce the viability of a hematopoietic tumor cell *in vitro* or *in vivo*, even in an immunocompetent animal, using the claimed methods. In other words, as long as one skilled in the art at the time of filing, using the teachings of Applicants’ specification, could reduce the viability of a hematopoietic tumor cell commensurate with the scope of the claimed

methods, the claims are enabled. There has been no evidence presented to the contrary. Therefore, a *prima facie* case for lack of enablement has not been made.

Applicants have demonstrated herein that one skilled in the art, upon review of Applicants' specification, would have been enabled at the time of filing to reduce the viability of a hematopoietic tumor cell(s) without undue experimentation, even in an individual such as a human, using methods that are the subject matter of the claims presented herein. In addition, Applicants have discussed articles that provide further evidence that following the teachings of Applicants' specification one skilled in the art at the time of filing would have been able to reduce the viability of a hematopoietic tumor cell(s) comprising administering a vesicular stomatitis virus to a tumor cell(s). Furthermore, based on the principles of the articles cited by the Examiner and as discussed above, the *in vitro* and *in vivo* results presented in Applicants' specification and in some of the above articles demonstrate that one skilled in the art would conclude that these results will reasonably correlate with results expected in other animals (including humans) with respect to reducing the viability of a hematopoietic tumor cell(s) comprising administering a vesicular stomatitis virus to a tumor cell(s) as claimed.

VIII. CONCLUSION

Reversal of all rejections is respectfully requested.

No fee, other than the fee prescribed by 37 CFR 41.20(b)(2) for filing an Appeal Brief and the extension of time fee, is believed necessary in connection with the filing of this Appeal Brief. If any additional fee is required, the Commissioner is hereby authorized to charge the amount of such fee to Deposit Account No. 50-1677.

Respectfully submitted,

/Douglas A. Golightly/

Douglas A. Golightly
Reg. No. 51,244
Agent for Appellant(s)

930 Clopper Road
Gaithersburg, MD 20878
Phone: (240) 631-2500 x3342
Facsimile: (240) 683-3794

IX. CLAIMS APPENDIX

1. (Previously presented) A method of reducing the viability of a tumor cell, comprising administering to the tumor cell a virus, such that the virus is delivered to the tumor cell,

wherein said virus is a vesicular stomatitis virus and said tumor cell is a hematopoietic tumor cell and

wherein the virus is contained in a cell infected with the virus and the administering comprises administering the virus-infected cell.

2-5. (Cancelled)

6. (Previously presented) The method of claim 1, wherein the hematopoietic tumor cell is a leukemia, a lymphoma, or a myeloma.

7. (Previously presented) The method of claim 1, wherein the hematopoietic tumor cell is a leukemia.

8. (Original) The method of claim 7, wherein the leukemia is acute myelogenous leukemia.

9. (Original) The method of claim 7, wherein the leukemia is chronic myelogenous leukemia.

10. (Original) The method of claim 7, wherein the leukemia is promyelocytic leukemia.

11. (Original) The method of claim 7, wherein the leukemia is T cell leukemia.

12. (Previously presented) The method of claim 1, wherein the hematopoietic tumor cell is a lymphoma.

13. (Previously presented) The method of claim 1, wherein the hematopoietic tumor cell is a myeloma.

14-18. (Cancelled)

19. (Original) The method of claim 1, wherein the tumor cell is PKR^{-/-}; STAT1^{-/-}; or both PKR^{-/-} and STAT1^{-/-}.

20-23. (Cancelled)

24. (Previously presented) The method of claim 1, further comprising administering interferon to the tumor cell prior to administering VSV, such that the interferon is delivered to the tumor cell.

25. (Previously presented) The method of claim 1, wherein the virus is unable to inactivate PKR activity within the tumor cell.

26. (Previously presented) The method of claim 1, wherein the virus is an attenuated strain of vesicular stomatitis virus.

27. (Previously presented) The method of claim 1, wherein the virus is vesicular stomatitis virus strain M1.

28. (Previously presented) The method of claim 1, wherein the virus is vesicular stomatitis virus strain M2.

29. (Previously presented) The method claim 1, wherein the virus is vesicular stomatitis virus strain M3.

30. (Previously presented) The method of claim 1, wherein the virus is vesicular stomatitis virus strain M4.

31. (Previously presented) The method of claim 1, wherein the virus is vesicular stomatitis virus strain M5.

32. (Previously presented) The method of claim 1, wherein the tumor cell is in a mammalian subject.

33. (Previously presented) The method of claim 32, wherein the mammalian subject is a human.

34. (Previously presented) The method of claim 32, wherein the administering comprises administering the virus-infected cell to the subject by a route selected from intratumorally, intravenously and intraperitoneally.

35. (Previously presented) A method of reducing the viability of a tumor cell within a population of cells comprising administering a vesicular stomatitis virus to the population of cells, such that the virus is delivered to the population of cells,

wherein the virus is contained in a cell infected with the virus and the administering comprises administering the virus-infected cell,

wherein the population of cells comprises hematopoietic tumor cells and non-tumor cells and

wherein the virus is able to selectively reduce the viability of the hematopoietic tumor cells.

36. (Original) The method of claim 35, wherein the virus is unable to inactivate PKR activity in the tumor cell.

37. (Previously presented) The method of claim 36, further comprising treating the population of cells with interferon prior to administering the virus.

38-63. (Cancelled)

64. (Previously presented) The method of claim 35, wherein the hematopoietic tumor cells are leukemia cells.

65. (Previously presented) The method of claim 64, wherein the leukemia cells are acute myelogenous leukemia cells.

66. (Previously presented) The method of claim 64, wherein the leukemia cells are chronic myelogenous leukemia cells.

67. (Previously presented) The method of claim 64, wherein the leukemia cells are promyelocytic leukemia cells.

68. (Previously presented) The method of claim 64, wherein the leukemia cells are T cell leukemia cells.

69. (Previously presented) The method of claim 35, wherein the hematopoietic tumor cells are lymphoma cells.

70. (Previously presented) The method of claim 35, wherein the hematopoietic tumor cells are myeloma cells.

71. (Previously presented) The method of claim 35, wherein the tumor cells are PKR^{-/-}; STAT1^{-/-}; or both PKR^{-/-} and STAT1^{-/-}.

72. (Previously presented) The method of claim 35, wherein the virus is an attenuated strain of vesicular stomatitis virus.

73. (Previously presented) The method of claim 35, wherein the virus is vesicular stomatitis virus strain M1.

74. (Previously presented) The method of claim 35, wherein the virus is vesicular stomatitis virus strain M2.

75. (Previously presented) The method of claim 35, wherein the virus is vesicular stomatitis virus strain M3.

76. (Previously presented) The method of claim 35, wherein the virus is vesicular stomatitis virus strain M4.

77. (Previously presented) The method of claim 35, wherein the virus is vesicular stomatitis virus strain M5.

78. (Previously presented) The method of claim 35, wherein the administering of the vesicular stomatitis virus to the population of cells is performed *in vitro*.

79. (Previously presented) The method of claim 32, wherein the mammalian subject is a non-human mammal.

80. (Previously presented) The method of claim 32, further comprising treating the mammalian subject with an interferon.

X. EVIDENCE APPENDIX

- Ahmed *et al.*, J. Virol. (2003) 77(8): 4646-4657.
 - First cited in Applicants' Reply of April 7, 2008
- Balachandran and Barber (IUBMB Life 50:135-138 (2000))
 - First cited in Applicants' Reply of August 2, 2007
- Bibby (Eur. J. Cancer 2004 40(6):852-857)
 - First cited in the February 7, 2007 Office Action
- Césaire *et al.* (Oncogene, 25:349-358 (2006))
 - First cited in Applicants' Reply of August 2, 2007
- Chu *et al.* (Section 19.2, para. bridging pp. 352-353)Cancer Principles & Practice of Oncology (2001; 6th edition; editors: DeVita VT, Jr, Hellman S, and Rosenberg SA)),
 - First cited in Applicants' Reply of November 26, 2004
- Desforges, *et al.*, Virus Res. (2001) 76(1): 87-102 (Abstract).
 - First cited in Applicants' Reply of September 16, 2008
- Ferran *et al.*, J. Virol. (1977) 71(1): 371-377.
 - First cited in Applicants' Reply of April 7, 2008
- Guide for Authors, Virology (as downloaded July 13, 2005)
 - First cited in Applicants' Reply of April 7, 2008
- Gura (Science, 1997, 278:1041-42)
 - First cited in the February 7, 2007 Office Action
- Instructions to Authors, Journal of Virology (Jan. 2005) 79(1): 1-16
 - First cited in Applicants' Reply of April 7, 2008
- Kelland (Eur. J. Cancer. 2004, 40(6):827-836)

- First cited in the February 7, 2007 Office Action
- Lichty *et al.* (Human Gene Therapy, 15:821-831 (2004))
 - First cited in Applicants' Reply of August 2, 2007
- Marcus *et al.*, J. Gen. Virol. (1980) 47(1): 89-96 (Abstract).
 - First cited in Applicants' Reply of April 7, 2008
- McCormick (U.S. Patent No. 5,677,178)
 - First cited in Applicants' Reply of October 21, 2002
- Nemunaitis *et al.* Journal of Clinical Oncology, 19(2):289-298 (2001)
 - First cited in Applicants' Reply of August 2, 2007
- Office Action, April 10, 2008, page 21, for U.S. Patent Application No. 11/685,483
 - First cited in Applicants' Reply of September 16, 2008
- Pasternak, *et al.*, Virology (1988) 166(2): 379-386 (Abstract).
 - First cited in Applicants' Reply of April 7, 2008
- Pecora (Pecora *et al.* J. of Clin. Oncol. 20(9):2251-2266 (2002))
 - First cited in Applicants' Reply of October 21, 2002
- Peterson *et al.* (Eur. J. Cancer 2004 40:837-844)
 - First cited in the February 7, 2007 Office Action
- Porosnicu *et al.* (Cancer Research 63:8366-8376 (2003))
 - First cited in Applicants' Reply of August 2, 2007
- Saijo *et al.* (Cancer Science 2004, 95(10):772-776)
 - First cited in the February 7, 2007 Office Action
- Schuh (Toxicologic Pathology 2004, 32(Suppl. 1):53-66)
 - First cited in the February 7, 2007 Office Action

- Stanners, *et al.*, Cell (1977) 11(2): 273-281 (Abstract).
 - First cited in Applicants' Reply of April 7, 2008
- Stojdl *et al.* 2000 (Nature Medicine, 6(7):821-825 (2000))
 - First cited in Applicants' Reply of August 2, 2007
- Stojdl *et al.* 2003 (Cancer Cell, 4:263-275 (2003))
 - First cited in Applicants' Reply of November 26, 2004
- Voskoglou-Nomikos *et al.* (Clin. Cancer Res. 2003 9:4227-4239)
 - First cited in the February 7, 2007 Office Action
- Wang *et al.* (Exp. Opin. Biol. Ther. 2001, 1(2):277-290)
 - First cited in the February 7, 2007 Office Action
- Winograd, *et al.*, In Vivo (1987) 1: 1-14
 - First cited in Applicants' Reply of November 26, 2004
- Winship *et al.*, J. Gen. Virol. (1984) 65: 843-847 (Abstract).
 - First cited in Applicants' Reply of April 7, 2008

Decisions

- *Ex parte Ayishi* (Appeal No. 2006-1608 before the BPAI, not binding precedent of the Board)
 - First cited in Applicants' Reply of April 7, 2008
- *Ex parte Boutin* (Appeal No. 2006-1879 before the BPAI, not binding precedent of the Board)
 - First cited in Applicants' Reply of April 7, 2008
- *Ex parte Saito and Zhao* (Appeal No. 2005-1442 before the BPAI, not binding precedent of the Board)
 - First cited in Applicants' Reply of April 7, 2008
- *In re Brana*, 51 F.3d 1560, 1567, 34 USPQ2d 1436, _____ (Fed. Cir. 1995)

- First cited in Applicants' Reply of October 21, 2002
- *In re Marzocchi*, 439 F.2d 220, 223, 169 USPQ 367, 369 (CCPA 1971)
 - First cited in Applicants' Reply of October 21, 2002

XI. RELATED PROCEEDINGS APPENDIX

None.

Ability of the Matrix Protein of Vesicular Stomatitis Virus To Suppress Beta Interferon Gene Expression Is Genetically Correlated with the Inhibition of Host RNA and Protein Synthesis

Maryam Ahmed,^{1,*} Margie O. McKenzie,¹ Shelby Puckett,¹ Michael Hohnacki,¹
Laurent Poliquin,² and Douglas S. Lyles¹

Department of Microbiology and Immunology, Wake Forest University School of Medicine, Winston-Salem, North Carolina 27157,¹ and Department of Biological Sciences, Université du Québec à Montréal, Montréal, Québec, Canada H3C 3P8²

Received 3 September 2002/Accepted 15 January 2003

The vesicular stomatitis virus (VSV) matrix (M) protein plays a major role in the virus-induced inhibition of host gene expression. It has been proposed that the inhibition of host gene expression by M protein is responsible for suppressing activation of host interferon gene expression. Most wild-type (wt) strains of VSV induce little if any interferon gene expression. Interferon-inducing mutants of VSV have been isolated previously, many of which contain mutations in their M proteins. However, it was not known whether these M protein mutations were responsible for the interferon-inducing phenotype of these viruses. Alternatively, mutations in other genes besides the M gene may enhance the ability of VSV to induce interferons. These hypotheses were tested by transfecting cells with mRNA expressing wt and mutant M proteins in the absence of other viral components and determining their ability to inhibit interferon gene expression. The M protein mutations were the M51R mutation originally found in the α 082 and T1026R1 mutant viruses, the double substitution V221F and S226R found in the TP3 mutant virus, and the triple substitution E213A, V221F, and S226R found in the TP2 mutant virus. wt M proteins suppressed expression of luciferase from the simian virus 40 promoter and from the beta interferon (IFN- β) promoter, while M proteins of interferon-inducing viruses were unable to inhibit luciferase expression from either promoter. The M genes of the interferon-inducing mutants of VSV were incorporated into the wt background of a recombinant VSV infectious cDNA clone. The resulting recombinant viruses were tested for their ability to activate interferon gene expression and for their ability to inhibit host RNA and protein synthesis. Each of the recombinant viruses containing M protein mutations induced expression of a luciferase reporter gene driven by the IFN- β promoter and induced production of interferon bioactivity more effectively than viruses containing wt M proteins. Furthermore, the M protein mutant viruses were defective in their ability to inhibit both host RNA synthesis and host protein synthesis. These data support the idea that wt M protein suppresses interferon gene expression through the general inhibition of host RNA and protein synthesis.

Virus infections usually trigger an antiviral response in host cells that functions to inhibit virus replication. As a result, most viruses have evolved mechanisms to suppress the antiviral response of the host. The balance between the ability of host cells to mount an antiviral response and the ability of the virus to suppress that response is a major determinant of the evolution of infection and viral tissue tropism in intact animal hosts (reviewed in reference 31). For many viruses, a major factor in the host antiviral response is the production of alpha and beta interferon (IFN- α and - β). Once IFNs are secreted by infected cells, signal transduction events are stimulated, both in the infected cells and in neighboring uninfected cells, which lead to the activation of genes whose products interfere with various steps in the viral life cycle (reviewed in reference 21). However, many viruses have evolved diverse mechanisms to combat the host defense mounted by IFNs. In general, these mechanisms can be divided into two types: those that inhibit the production of IFNs and those that inhibit the response to IFNs. Vesicular

stomatitis virus (VSV), the prototype rhabdovirus, is a classic example of a virus that inhibits the production of IFNs (33). The goal of the experiments presented here was to determine whether the activity of the viral matrix (M) protein aids in the suppression of IFN gene expression during VSV infection.

The M protein of VSV plays a major role in virus assembly by binding the viral nucleocapsid to the cytoplasmic surface of the host plasma membrane during the budding process (17, 18, 28) and by inducing budding of virus envelopes (22, 23, 25). However, M protein is also responsible for many of the cytopathic effects associated with VSV infection. These include the characteristic rounding of cells, as well as the shutoff of host-directed gene expression (reviewed in reference 31). The ability of M protein to repress host gene expression is genetically separable from its viral assembly function (6, 11, 26). Furthermore, M protein is capable of inhibiting host gene expression independently of other viral components (5, 16, 36). This inhibition occurs at the level of host transcription, as well as nuclear-cytoplasmic transport of host RNAs and proteins (1, 5, 24, 37, 39). M protein also plays a major role in the inhibition of host translation (19, 25, 27). However, M protein is not able to inhibit translation in transfected cells in the absence of other

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Wake Forest University School of Medicine, Medical Center Blvd., Winston-Salem, NC 27157. Phone: (336) 716-3301. Fax: (336) 716-9928. E-mail: mahmed@wfuolm.edu.

viral components (4). Therefore, it is likely that the inhibition of host translation in VSV-infected cells is due to the combined effects of M protein and additional viral factors.

It has been proposed that the ability of M protein to inhibit host gene expression is responsible for the ability of VSV to suppress activation of host IFN gene expression (reviewed in reference 31). According to this model, there must be other products of virus infection, such as viral double-stranded RNA (dsRNA), that activate IFN gene expression, which is then suppressed by the activity of M protein. In support of this idea, transfection experiments have shown that M protein inhibits expression of a reporter gene from a plasmid containing the IFN- β promoter as effectively as it inhibits expression from other promoters (16). Despite the extensive evidence that M protein can inhibit host gene expression in the absence of other viral components, the role of M protein versus other viral components in the shutoff of host gene expression in the context of a viral infection has been questioned (40). In addition, it has been proposed that M protein plays little, if any, role in the suppression of IFN production by VSV (34).

Earlier studies have demonstrated the feasibility of isolating VSV mutants with strong IFN-inducing phenotypes (19, 34). Many of these IFN-inducing mutants contain point mutations in their M proteins (13, 34). However, it was not known whether the M protein mutations were responsible for the ability of these viruses to induce IFN. Alternatively, it has been proposed that mutations in other genes besides the M gene may account for their IFN-inducing phenotype (34). In this paper, mutant M proteins from these IFN-inducing viruses were used to resolve the question of whether M protein is the VSV factor that suppresses IFN induction during the virus infection. Furthermore, we tested whether the ability of M protein to inhibit IFN induction is due to its potent ability to shut off host gene expression. These hypotheses were tested in the experiments presented here by transfecting cells with M mRNA expressing wild-type (wt) M proteins or mutant M proteins from the IFN-inducing viruses and determining their ability to inhibit IFN gene expression. Results indicated that wt M proteins effectively suppressed luciferase expression from plasmids containing either the simian virus 40 (SV40) or IFN- β promoters, while M proteins of IFN-inducing viruses were unable to inhibit luciferase expression from either promoter. The M genes of several of these IFN-inducing mutants were incorporated into the wt background of an infectious VSV cDNA clone. Each of the recombinant viruses containing mutant M proteins induced expression of a luciferase reporter gene driven by the IFN- β promoter and induced the production of IFN bioactivity more effectively than viruses containing wt M proteins. These results indicate that M protein plays a major role in the inhibition of host IFN gene expression in VSV-infected cells. Furthermore, the M protein mutant viruses were defective in their ability to inhibit both host RNA synthesis and host protein synthesis. Thus, the IFN-inducing phenotype of the viruses containing M protein mutations was genetically correlated with a defect in their ability to inhibit host gene expression, suggesting that the suppression of IFN activity in VSV-infected cells is due in part to a global inhibition of host gene expression by M protein.

MATERIALS AND METHODS

Cells and viruses. HeLa cells and PC-3 prostate cells were from the American Type Culture Collection, wt VSV (Indiana serotype, Orsay strain) and the M protein mutant r082 (11) were grown in BHK cells as described previously (32). The recombinant viruses, rwt and rM18r, contain the San Juan strain of M protein and were isolated from infectious VSV cDNA clones as described elsewhere (27). Plasmids containing cDNA copies of the M genes of the wt HR strain and the T1026R1, TP2, and TP3 mutant viruses have been described previously (13). The M genes were modified by PCR with *Pwo* DNA polymerase (Boehringer-Mannheim, Inc.) using the primers 5'-GGGCTTAAGGAAGATTCTCGTCTG3' and 5'-TTTGGCGCGCAATTAGGAGAGAC3'. The PCR products were digested with *Afl*II and *Bst*HI and inserted into the infectious VSV cDNA clone as described previously (27). The recombinant viruses isolated from these cDNA clones were designated rHR-M, r1026-M, rTP2-M, and rTP3-M viruses. All viruses were plaque isolated twice in BHK cells, and the sequences of the M genes were confirmed by reverse transcription-PCR and automated DNA sequencing as described elsewhere (27).

Plasmids and in vitro transcription of mRNA. The pBlux plasmid contains the firefly luciferase gene under control of the IFN- β promoter (35), and the pGL3 control vector expresses luciferase constitutively from the SV40 promoter (Promega). The plasmid pSD-OM, used for in vitro transcription of mRNA encoding wt M protein (Orsay strain) together with a 3' poly(A) sequence, has been described previously (4). The M gene cDNAs from wt HR, T1026R1, TP2, and TP3 viruses were modified by PCR with *Pwo* DNA polymerase by using the primers described previously (6). The PCR products were digested with *Hind*III and were cloned into the pSD42 vector for in vitro transcription of wt HR and mutant M mRNAs. In the in vitro transcription reactions, M mRNAs containing 5' caps and 3' poly(A) were synthesized in the presence of the cap analog mG(5')ppp(5')G from linearized plasmid DNA by the bacteriophage SP6 RNA polymerase (Message Machine; Ambion, Inc.).

Transfections and luciferase assays. HeLa cells in 35-mm-diameter dishes (or six-well plates) were transfected using Lipofectin reagent (GIBCO-BRL) according to the manufacturer's instructions. To determine the effect of M protein on expression of luciferase from the SV40 promoter, cells were transfected with various amounts of in vitro-transcribed M mRNA together with 250 ng of pGL3 plasmid DNA and various amounts of yeast RNA to normalize RNA levels to 750 ng. At 16 h posttransfection, cells were washed with phosphate-buffered saline (PBS) and harvested. Luciferase activity was determined using the Promega luciferase assay system.

To determine the effect of wt and mutant M proteins on expression of luciferase from the IFN- β promoter, cells were transfected with either 100 ng of wt HR, TP2, and TP3 M mRNAs or 300 ng of T1026 M mRNA together with 1 μ g of pBlux. At 16 h posttransfection, cells were washed with PBS and harvested, and luciferase activity was measured. Transfections were carried out both in the presence and absence of poly(I)-poly(C). However, in most cases, the transfection protocol itself partially induced the IFN promoter, and the addition of dsRNA had little if any effect in activation of the IFN promoter.

To determine the effect of wt and M protein mutant viruses on activation of the IFN- β promoter, HeLa cells in 35-mm dishes were transfected with 1 μ g of pBlux plasmid DNA. At 24 h posttransfection, cells were infected with viruses containing wt or mutant M proteins at a multiplicity of 20 PFU/cell. Cells were mock infected as negative controls or were treated with poly(I)-poly(C) (200 μ g/ml; Sigma Chemical Co.) at 24 h posttransfection as positive controls. Cells were harvested at 3, 6, and 9 h postinfection, and luciferase activity was measured.

IFN bioassay. To determine the IFN activity produced by cells infected with wt and mutant viruses, supernatants (100 μ l) were collected from HeLa and PC-3 cells infected with wt and mutant viruses at the times indicated below in Fig. 5. Infectious virus was inactivated by acid treatment, the acid was neutralized, and serial dilutions were incubated with HeLa cells in 96-well plates overnight at 37°C. As a standard, cells were incubated with serial fivefold dilutions of IFN (Universal type I IFN; PBL Biomedical Laboratories, New Brunswick, N.J.). The samples were aspirated, and cells were challenged with wt VSV at 2.24×10^4 PFU/ml in 100 μ l of medium. Controls included cells infected with VSV alone and cells that were not challenged with VSV. Cells were incubated overnight at 37°C, medium was aspirated, and cells were fixed with 95% ethanol. Cells were then stained with a 0.1% crystal violet solution in methanol. Absorbance was read at 550 nm on an ELISA reader.

Radiolabeling of infected and transfected cells. To analyze host and viral protein synthesis during virus infections, HeLa cells in 35-mm dishes were infected with viruses containing wt or mutant M proteins at a multiplicity of 20 PFU/cell in Dulbecco's modified essential medium (DMEM) with 2% fetal calf

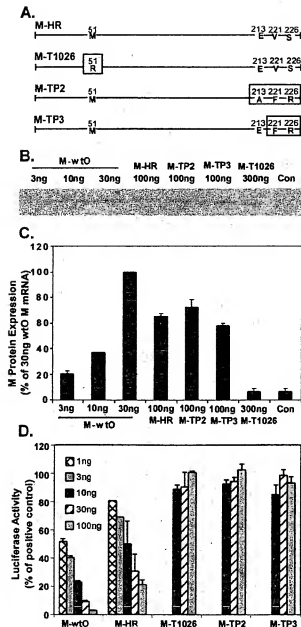


FIG. 1. Effect of wt and mutant M proteins on expression of luciferase from the SV40 promoter. (A) Diagram representing sequences of M proteins from IFN-inducing mutant viruses of the HR strain of VSV. M-HR is the wt M protein of the HR strain. Mutations in the M-T1026, M-TP2, and M-TP3 proteins are indicated by boxes. (B) Representative image of M proteins expressed from cells transfected with wt and mutant M mRNAs. L cells were transfected with the indicated amounts of wt and mutant M mRNAs for 5 h. Cells transfected with pGL3 plasmid DNA alone were used as a negative control. Cells were labeled with [35 S]methionine (200 μ Ci/ml) for 1 h, and cell extracts were prepared. Extracts were immunoprecipitated with the anti-M protein monoclonal antibody 23H12 and processed for SDS-PAGE and phosphorimaging. (C) Quantitation of labeled M proteins. Results are expressed as the percentage of M protein expressed in cells transfected with 30 ng of wtO mRNA. Data are the means \pm standard errors of the means for four experiments. (D) Effect of wt and mutant M proteins on expression of luciferase from the SV40 promoter. HeLa cells were transfected with the indicated amounts of in vitro-transcribed M mRNA together with 250 ng of pGL3 plasmid DNA con-

taining a luciferase gene driven by the SV40 promoter. At 24 h posttransfection, cells were harvested and luciferase activity was measured. Data are presented as the percentage of the activity of controls transfected with pGL3 plasmid DNA in the absence of M mRNA and are the means \pm standard errors of the means for eight independent experiments.

serum (FCS). At 4, 8, and 12 h postinfection, cells were labeled with a 15-min pulse of [35 S]methionine (100 μ Ci/ml) in a total of 0.3 ml of methionine-free medium. Cells were washed with PBS and harvested in radioimmunoprecipitation assay (RIPA) buffer (0.15 M NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 10 mM Tris [pH 7.4]). Cell extracts were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and phosphorimaging as described elsewhere (27).

To determine the amount of M protein expressed by mRNAs encoding wt or mutant M proteins, cells were transfected with various amounts of M mRNA or with 250 ng of pGL3 vector alone. At 5 h posttransfection, cells were washed with PBS and harvested in RIPA buffer. Cell extracts were immunoprecipitated with the anti-M protein monoclonal antibody, 23H12, and processed for electrophoresis as described previously (6). Data were quantitated by phosphorimaging.

RNA synthesis in infected cells. HeLa cells were infected with viruses containing wt or mutant M proteins at a multiplicity of 20 PFU/cell in DMEM plus 2% FCS at 37°C or were mock infected as a control. The virus was allowed to adsorb for 1 h, and cells were fed with medium containing 2% FCS. Parallel samples were incubated in the presence of actinomycin D (total concentration of 5 μ M/ml). At 2, 4, and 6 h postinfection, cells were labeled with [3 H]uridine (20 μ Ci/ml) for 30 min, washed in PBS, and harvested. Cells were resuspended in SDS-lysis buffer containing RNase-proteinase degrader (Invitrogen), and DNA was sheared with a 20-gauge needle. Samples were then precipitated with 7% trichloroacetic acid on ice and washed twice with 7% trichloroacetic acid. Acid-precipitable radioactivity was measured by scintillation counting.

Growth curve assay. HeLa cells in 35-mm dishes were infected with viruses containing wt or mutant M proteins (multiplicity of infection = 10 PFU/cell) in DMEM containing 2% FCS. At 1 h postinfection, the medium was removed and cells were washed twice with PBS and then fed with 2 ml of DMEM containing 10% FCS. At the indicated times postinfection, 100 μ l of medium was removed from the dishes and stored at -70°C. The yield of virus was determined by plaque assays on BHK cells and was expressed as PFU per milliliter.

RESULTS

Effect of mutant M proteins expressed from transfected mRNA on expression from the SV40 promoter. To determine whether the ability of M protein to inhibit host gene expression is responsible for the ability of the virus to suppress activation of IFN gene expression, we asked if the mutant M proteins from the previously isolated IFN-inducing VSV mutants (19) were defective in their ability to inhibit host gene expression. Therefore, we tested the effect of wt and mutant M proteins expressed from transfected M mRNA, in the absence of other viral components, on expression of luciferase from a plasmid containing the SV40 promoter. These IFN-inducing viruses are derived from the HR strain of VSV, and their M protein mutations are depicted in the diagram in Fig. 1A. The M protein of the IFN-inducing mutant T1026R1 virus (M-T1026) has a substitution of arginine for methionine at position 51 (M51R mutation) of the 229-amino-acid M protein (13, 16). This mutation has been shown previously to render the M protein defective in its ability to inhibit host gene expression (1, 6, 16, 37, 45). M-TP2 and M-TP3 are the M proteins from the TP2 and TP3 mutant viruses, respectively, which were independently isolated from the HR strain based on their IFN-inducing phenotypes (19). These M proteins contain the double substitution V221F and S226R (TP3) and the triple substitution E213A, V221F, and S226R (TP2), which are near

taining a luciferase gene driven by the SV40 promoter. At 24 h posttransfection, cells were harvested and luciferase activity was measured. Data are presented as the percentage of the activity of controls transfected with pGL3 plasmid DNA in the absence of M mRNA and are the means \pm standard errors of the means for eight independent experiments.

the carboxy terminus of the 229-amino-acid M protein (13). The ability of these mutant M proteins to inhibit host gene expression in the absence of other viral components had not been tested previously.

M protein inhibits its own transcription when expressed from DNA vectors that depend on host cell transcription, making it difficult to express from recombinant plasmid DNA vectors (5, 7, 29). To circumvent this problem, M protein can be more effectively expressed by transfecting cells with in vitro-transcribed M mRNA instead of plasmid DNA (4). This is because M protein does not inhibit translation of transfected mRNAs in the absence of other viral components. In fact, wt M protein actually stimulates translation of transfected mRNAs, including its own mRNA (4). This leads to higher levels of expression of wt M protein compared to mutant M proteins when cells are transfected with equivalent amounts of M mRNAs (as shown below and in reference 32).

The relative levels of expression from transfected mRNAs encoding M proteins derived from the HR strain (M-HR, M-TP2, M-TP3, and M-T1026) were compared to those of the M protein of the Orsay strain of VSV (M-wtO), which we had studied previously (1, 5, 6). Cells were transfected with 100 ng of wtHR, TP2, or TP3 M mRNAs or 300 ng of T1026 M mRNA and compared to cells transfected with 3, 10, or 30 ng of wtO M mRNA. At 5 h posttransfection, cells were radiolabeled with [³⁵S]methionine for 1 h and lysed. Lysates were immunoprecipitated with the anti-M monoclonal antibody, 23H12, and processed for electrophoresis and phosphorescence imaging. Similar results were obtained in L cells and HeLa cells. However, only the data from L cells were quantitated due to the fact that immunoprecipitates from HeLa cell lysates contained high levels of background proteins, making it difficult to quantitate the amount of M protein expressed from M mRNA. An image of the labeled M protein bands is shown in Fig. 1B. The wt and mutant M proteins synthesized by each of the M mRNAs were quantitated and are shown as a percentage of the M protein expressed in cells transfected with 30 ng of wtO M mRNA (Fig. 1C). The M proteins derived from the HR strain were less effectively expressed than the wtO M protein, so that at least three times more wtHR, TP2, or TP3 M mRNA was needed to achieve levels of M protein expression comparable to that of wtO M protein. The amount of M protein obtained by transfecting 300 ng of T1026 M mRNA was close to background levels. Therefore, it appears that the T1026 M protein was not expressed efficiently from transfected mRNA. In contrast, the TP2 and TP3 M proteins were expressed as efficiently as wtHR M protein. The low level of detection of the T1026 M protein is not likely to be due to lack of antibody reactivity with the mutant M protein. Even though the M51R mutation in this M protein is near the epitope recognized by this antibody against M protein (37), the M51R mutant M protein of the Orsay strain is immunoprecipitated as efficiently as wt M protein (37; H. Yuan and D. S. Lyles, unpublished data).

To determine the ability of wt and mutant M proteins to inhibit host-directed gene expression, HeLa cells were transfected with 250 ng of plasmid DNA encoding luciferase expressed from the SV40 promoter, together with varying amounts of wt or mutant M mRNA. At 24 h posttransfection, cell extracts were prepared, and luciferase activity was mea-

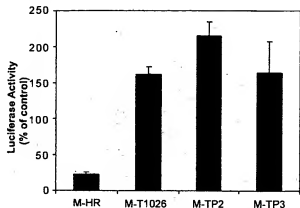


FIG. 2. Effect of wt and mutant M proteins on expression of luciferase from the IFN- β promoter. HeLa cells were transfected with 1 μ g of pBluc encoding luciferase expressed from the IFN- β promoter, together with 100 ng of wt, TP2, and TP3 M mRNA or 300 ng of T1026 M mRNA. At 16 h posttransfection, cell extracts were prepared, and luciferase activity was measured. Luciferase activities are expressed as a percentage of the activity in cells transfected with pBluc alone and are the means \pm standard errors of the means for four independent experiments.

sured. Data are expressed as a percentage of the luciferase activity obtained from cells transfected with the luciferase plasmid in the absence of M mRNA (Fig. 1D). The wtO M protein inhibited luciferase expression from the plasmid containing the SV40 promoter with approximately 50% inhibition when cells were transfected with 1 to 3 ng of M mRNA. We have previously shown by nuclear runoff experiments that M protein inhibits expression from the SV40 promoter at the transcriptional level (1, 5). It is also possible that inhibition of nuclear-cytoplasmic mRNA transport contributes to the inhibition of luciferase expression (5, 24, 37, 39). Similar to wtO M protein, the wt HR M protein also inhibited luciferase expression, although 10 ng of HR M mRNA was required to achieve 50% inhibition. Since more than threefold more HR M mRNA was required to give levels of expression equivalent to those of wtO M mRNA (Fig. 1C), these data indicate that the potency of the wtHR M protein is similar to that of the wtO M protein, when the relative expression levels are considered. All of the M protein mutants of the HR strain (TP2, TP3, and T1026) were defective in their ability to inhibit luciferase expression. In fact, luciferase activity remained constant in cells transfected with concentrations of each of the mutant M mRNAs that were 10-fold higher than those used with the wt M mRNAs (Fig. 1D). In the case of the TP2 and TP3 M proteins, the inability to inhibit luciferase expression cannot be accounted for by low levels of expression, since they were expressed as efficiently as the wtHR M protein (Fig. 1C). In the case of the T1026 M protein, the low level of expression could be responsible, in part, for its failure to inhibit luciferase expression. Taken together these results indicate that the mutant M proteins are defective in their ability to inhibit host-directed gene expression when expressed in transfected cells in the absence of other viral gene products.

Mutant M proteins induce expression of luciferase from the

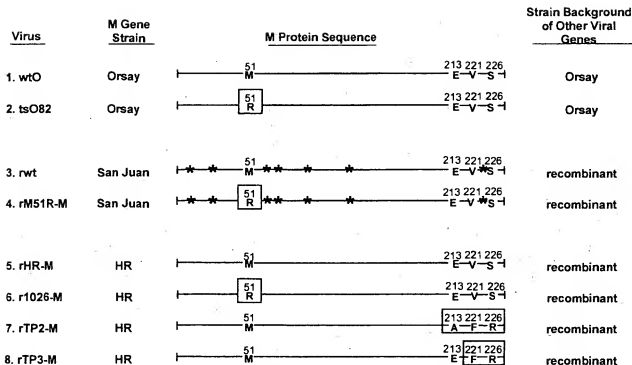


FIG. 3. Viruses used in this study. The diagram represents the sequences of the M proteins of the viruses used in this study. Mutations in the M proteins are indicated by boxes. The tsO82 virus (number 2) is a naturally occurring mutant of the Orsay strain of VSV (number 1) containing the M51R mutation (11). The remaining viruses are recombinants isolated from VSV infectious cDNA clones, which differ only in their M genes. The M genes of the original recombinant wt (rwt) virus (3) and rM51R-M mutant (4) are derived from the San Juan strain (27, 42). The viruses containing the wt M protein from the HR strain (rHR-M virus) (5), the M51R mutation in the HR M protein (r1026-M virus) (6), and the TP2 and TP3 mutations in the HR M protein (7 and 8) were generated for this study. Sites of amino acid differences between the San Juan and HR strains are indicated by asterisks.

IFN- β promoter. Data in Fig. 1 indicate that mutant M proteins from IFN-inducing VSV mutants are defective in their ability to shut off luciferase gene expression driven from the SV40 promoter. In a similar assay, we also tested the effect of wt and mutant M proteins on expression of luciferase from a plasmid containing the IFN- β promoter. HeLa cells were transfected with 1 μ g of plasmid DNA encoding luciferase expressed from the IFN- β promoter (pBlux), together with 100 ng of wtHR, TP2, and TP3 M mRNAs or 300 ng of T1026 M mRNA. At 16 h posttransfection, cell extracts were prepared, and luciferase activity was measured. In order to compare data between different experiments, luciferase activities are expressed as a percentage of the activity in cells transfected with pBlux alone. Results in Fig. 2 show that the wt M protein of the HR strain inhibited expression of luciferase from the plasmid expressing the IFN- β promoter. This result is similar to previous results obtained using a different reporter gene driven by the IFN- β promoter (16). However, each of the mutant M proteins failed to inhibit luciferase expression from the plasmid containing the IFN- β promoter. In fact, luciferase was expressed at somewhat higher levels in cells expressing mutant M proteins than in control cells. Such stimulation of gene expression by mutant M proteins has not been observed previously (2, 6). However, the basis for this effect has not been explored. The important conclusion from Fig. 2 is that the M proteins of

these IFN-inducing VSV mutants are defective in their ability to inhibit luciferase activity driven by the IFN- β promoter, similar to their inability to inhibit the activity from the SV40 promoter (Fig. 1).

M protein mutations contribute to defects in IFN suppression in the context of a virus infection. The M genes from the IFN-inducing mutants of VSV were incorporated into the wt background of a recombinant VSV infectious cDNA clone to determine whether the M protein mutations contribute to their IFN-inducing phenotype in the context of the virus infection. In addition to the wt and mutant M proteins derived from the HR strain of VSV, we also tested the effects of mutations in M genes of additional virus strains (Orsay and San Juan). The viruses used in our study are diagrammed in Fig. 3. Viruses 1 and 2 are naturally occurring viruses derived from the Orsay strain of VSV, and the remaining viruses are recombinant viruses isolated from VSV infectious cDNA clones. Virus 1 is the wt Orsay strain (wtO), and virus 2 is the tsO82 mutant derived from wtO virus, which was shown previously to induce higher levels of IFN production than wtO virus (34). tsO82 virus contains the methionine-to-arginine substitution in position 51 (M51R substitution) in the M protein sequence (11). The M51R mutation was introduced into the wt background of our recombinant wt virus (rwt virus; number 3 in Fig. 3) to generate rM51R-M virus (number 4), using infectious cDNA clones

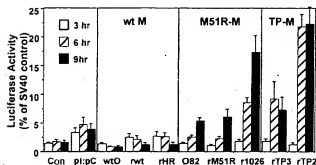


FIG. 4. Effect of viruses containing wt or mutant M proteins on the activity of the IFN- β promoter. HeLa cells were transfected with 1 μ g of pBlux plasmid DNA encoding luciferase under control of the IFN- β promoter. At 24 h posttransfection, cells were infected with viruses containing wt or mutant M proteins at a multiplicity of 20 PFU/cell. Cells were harvested at 3, 6, and 9 h postinfection, and luciferase activity was determined. Cells were transfected with pBlux DNA and mock infected as negative controls (Con), and cells were transfected with pBlux DNA and then treated with poly(I)-poly(C) (pI-pC) as positive controls. Data are expressed as a percentage of the luciferase activity expressed by uninfected cells transfected with 250 ng of pGL3 plasmid DNA to detect constitutive luciferase activity from the SV40 promoter. Data shown are means \pm standard errors of the means for four independent experiments.

modified slightly (27) from the one described by Whelan et al. (42). The M proteins of these viruses are derived from the San Juan strain of VSV. The San Juan M protein differs from the HR M protein by seven amino acid substitutions, as indicated in Fig. 3.

Viruses 5 to 8 are recombinant viruses containing M proteins derived from the HR strain of VSV (as shown in Fig. 1A). Virus 5 (rHR-M virus) contains the wtHR M protein, and virus 6 (r1026-M virus) contains the M protein of the IFN-inducing mutant virus T1026R1. As mentioned previously, the M protein of r1026-M contains the same M51R mutation found in the rO82 and rM51R-M viruses. Viruses 7 and 8 contain the M proteins of the TP2 and TP3 mutant viruses, which contain mutations near the carboxy terminus of the M protein (13).

The effect of M protein mutations on the ability of these viruses to stimulate IFN gene expression was determined by transfecting HeLa cells with the plasmid encoding luciferase under control of the IFN- β promoter (pBlux). At 24 h posttransfection, cells were infected with viruses containing wt or mutant M proteins. Cells were harvested at 3, 6, or 9 h postinfection, and lysates were tested for luciferase activity. Data are expressed as a percentage of the luciferase activity in uninfected cells transfected with a control plasmid in which luciferase was expressed from the SV40 promoter (Fig. 4). Other controls included cells transfected with pBlux alone to determine unstimulated luciferase levels and cells treated with the dsRNA analog poly(I)-poly(C) to stimulate IFN gene expression.

All of the viruses containing wt M proteins (wtO, rwt, and rHR-M viruses) were unable to activate IFN gene expression, as demonstrated by little if any increase in luciferase levels over those of the negative control. In contrast, all of the viruses containing the M51R M protein mutation (rO82, rM51R-M, and r1026-M viruses) induced luciferase activity to levels as

high as or higher than that of the positive control treated with poly(I)-poly(C). Likewise, the rTP2-M and rTP3-M viruses, containing the carboxy-terminal substitutions in M protein, induced high levels of luciferase expression. Quantitatively, luciferase activity in cells infected with M protein mutant viruses was stimulated 2- to 10-fold over that seen with viruses containing wt M proteins. Pairwise comparison of recombinant viruses containing wt versus mutant M proteins derived from the same virus strain indicated that mutations in M protein are responsible for the IFN-inducing phenotypes of the recombinant viruses. For example, rM51R-M virus induced higher levels of luciferase than its wt control, rwt virus, and r1026-M virus induced higher levels of luciferase than its wt control, rHR-M virus.

The principal conclusion to be drawn from Fig. 4 is that viruses with wt M proteins inhibit expression of luciferase from the IFN promoter, while viruses with mutant M proteins activate luciferase expression. However, the data also show that differences in the virus strains from which the M proteins were derived play a role in dictating the amount of stimulation of IFN gene expression by M protein mutant viruses. For example, r1026-M virus, which contains the M51R mutation in the M gene from the HR strain, induced higher levels of luciferase than the rM51R-M virus, which contains the same M51R mutation in the M gene from the San Juan strain. The M proteins of these two recombinant viruses differ by seven amino acid substitutions, but the other viral genes besides the M gene are identical. Thus, the strain differences in the M proteins of these two viruses are responsible for the difference in induction of luciferase expression.

To confirm the results of the luciferase assay, we analyzed the IFN activity produced by cells infected with wt and mutant M protein viruses by an IFN bioassay. This assay is based on the reduction of VSV cytopathic effect by supernatants collected from infected cells. HeLa cells were infected with wt and mutant M protein viruses, and aliquots of the supernatant media at 12, 24, and 36 h postinfection were tested for IFN activity (Fig. 5A). The rTP2-M and rTP3-M viruses, containing carboxy-terminal mutations in the M gene, induced IFN activity in HeLa cells (Fig. 5A). However, the r1026-M virus, containing the M51R mutation, and the rHR-M virus, containing wt M protein, did not induce detectable IFN activity in HeLa cells. We noted that the rTP2-M and rTP3-M viruses required approximately 24 h to induce detectable levels of IFN activity. By this time, HeLa cells infected with the r1026-M virus were already dead due to virus-induced cellular apoptosis, similar to previous data (27). Therefore, we tested the ability of wt and M51R mutant viruses to induce IFN activity in PC-3 cells, a human prostate tumor cell line that is more resistant to VSV-induced killing (unpublished data). Results in Fig. 5B indicate that both viruses containing wt M proteins (wtO and rwt) were unable to induce IFN activity in PC-3 cells. However, the rO82, rM51R-M, and r1026-M viruses containing the M51R mutation in M proteins from different virus strains induced IFN activity to varying levels. Therefore, the overall conclusion from these data is that viruses containing wt M proteins are effective suppressors of IFN activity, while viruses with M protein mutations induce IFN activity. Once again, M proteins derived from different virus strains appear to play a role in dictating the degree of IFN activity induced by viruses with M

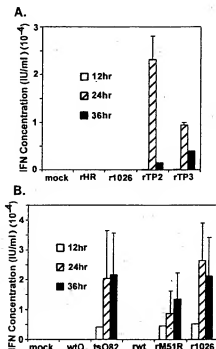


FIG. 5. IFN bioactivity produced by cells infected with wt and mutant M protein viruses. HeLa cells were incubated overnight at 37°C with serial dilutions of supernatants (100 μ l) collected from HeLa (A) and PC-3 (B) cells infected with wt and mutant viruses. The samples were aspirated, and cells were challenged with wt VSV at 2.24×10^4 PFU/ml in 100 μ l of medium. Cells were incubated overnight at 37°C, medium was aspirated, and cells were fixed and stained with crystal violet. Absorbance was read at 550 nm on an ELISA reader. The IFN concentration (in international units per milliliter) was quantitated by comparing results to those in cells incubated with serial fivefold dilutions of an IFN standard. Data shown are means \pm standard errors of the means for three independent experiments.

protein mutations. Not only did the r1026-M virus induce higher levels of luciferase expression from the IFN- β promoter than the rM51R-M virus (Fig. 4), it also induced higher levels of IFN activity in the bioassay (Fig. 5B).

M protein mutant viruses are defective at inhibiting host RNA and protein synthesis. The data in Fig. 4 and 5 indicate that M protein mutations are responsible for the ability of the recombinant M protein mutant viruses to activate IFN gene expression. Therefore, we can conclude that M protein mutations contribute to the IFN-inducing phenotypes of the original mutant viruses from which these M proteins were derived. The data in Fig. 1 and 2 suggest that the IFN-inducing phenotype of these viruses is due to defects in the inhibition of host gene expression. To test this hypothesis, synthesis of host RNA and proteins in cells infected with wt and M protein mutant viruses was determined by pulse-labeling experiments. Cells were infected with wt and mutant viruses and labeled with [3 H]uridine at 2, 4, and 6 h postinfection to determine the ratio of viral and host RNA synthesis. The time of the pulse (30 min) was short compared to the RNA turnover rate, so that labeling primarily reflected the rates of synthesis rather than turnover. Cells were lysed, and trichloroacetic acid-insoluble radioactivity was measured to determine the total cellular RNA synthesis (host plus

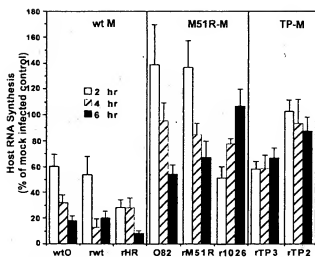


FIG. 6. Inhibition of host RNA synthesis by viruses containing wt or mutant M proteins. HeLa cells were infected with viruses containing wt or mutant M proteins at a multiplicity of 20 PFU/cell. At 2, 4, and 6 h postinfection, cells were labeled with [3 H]uridine (20 μ Ci/ml) for 30 min. Cells were lysed in SDS-lysis buffer, and aliquots were precipitated with trichloroacetic acid to measure acid-insoluble radioactivity. Parallel samples were incubated in the presence of actinomycin D, so that only viral RNA would be labeled. The rate of host RNA synthesis was calculated by subtracting the radioactivity in viral RNA from the total radioactivity. Data are expressed as a percentage of the uninfected cell control and are means \pm standard errors of the means for five experiments.

viral). Parallel samples were treated with actinomycin D to inhibit host RNA synthesis. In these samples only viral RNA would be labeled, since actinomycin D does not affect viral RNA synthesis. Host RNA synthesis was calculated by subtracting radioactivity in samples treated with actinomycin D from the total radioactivity in the absence of actinomycin D, and it is expressed as a percentage of the mock-infected controls (Fig. 6). Viruses with wt M proteins effectively inhibited host RNA synthesis, so that by 6 h postinfection levels of host RNA synthesis were 10 to 20% of controls. However, all of the M protein mutant viruses were defective in their ability to inhibit host RNA synthesis. Comparison of recombinant M protein mutant viruses with their isogenic counterparts with wt M proteins (rM51R-M versus rwt viruses, and r1026-M, rTP2-M, or rTP3-M versus rHR-M viruses) shows that M protein plays a major role in the inhibition of host RNA synthesis at early times postinfection.

The data in Fig. 6 also show that M protein mutants from different virus strains have slightly different effects on host RNA synthesis, similar to their differences in IFN gene induction. The tsO82 and rM51R-M viruses actually stimulated host RNA synthesis at early times postinfection (2 h), which then declined to about 60 to 70% of control by 6 h. In contrast, the r1026-M virus had an unusual effect in that host RNA synthesis decreased to 60% of control at 2 h postinfection, but host RNA synthesis increased over the time course of the experiment to 100% of control by 6 h postinfection. Cells infected with the rTP3-M and rTP2-M viruses maintained a constant level of

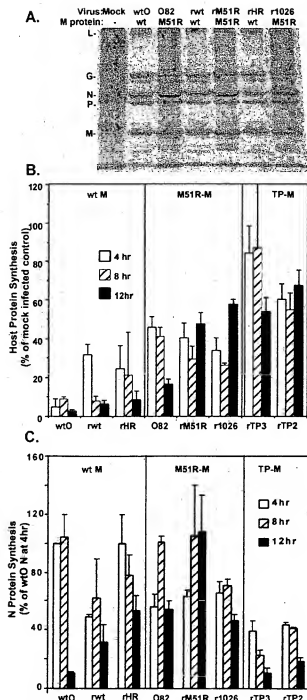


FIG. 7. Inhibition of host protein synthesis by viruses containing wt or mutant M proteins. HeLa cells were infected with viruses containing wt or mutant M proteins at a multiplicity of 20 PFU/cell or were mock infected as a control. Cells were labeled with a 15-min pulse of [35 S]methionine (100 μ Ci/ml) at 4, 8, and 12 h postinfection. Lysates were subjected to SDS-PAGE, and labeled proteins were quantitated by phosphorimaging. (A) Representative image from analysis of viruses containing wt or M51R mutant M proteins at 8 h postinfection. Positions of viral proteins are indicated on the left. (B) Host protein synthesis was determined from images similar to that in panel A in regions of the gel devoid of viral proteins between the L and G proteins and between the P and M proteins. Results are shown as a

percentage of the mock-infected control and are the mean \pm standard error of the mean of four independent experiments. (C) Effect of M protein mutations on viral protein synthesis. HeLa cells infected with viruses containing wt or mutant M proteins were labeled with [35 S]methionine, and the labeled proteins were analyzed by SDS-PAGE and phosphorimaging as described in the legend for Fig. 4. The labeled M proteins in images similar to those shown in Fig. 4A were quantitated and are expressed as a percentage of the wtO M protein labeled at 4 h postinfection. Data are the mean \pm standard error of the mean of four experiments.

RNA synthesis over the time course at around 60 and 90% of controls, respectively.

The inhibition of host gene expression in VSV-infected cells also involves an inhibition of host protein synthesis. The inhibition of host protein synthesis is not due to depletion of cellular mRNAs as a result of the inhibition of host transcription or transport. In fact, the cytoplasm of infected cells contains normal amounts of cellular mRNAs that can be effectively translated *in vitro* (30). Instead, the inhibition is due to inactivation of host translation factors (10, 14). To determine the ability of wt and mutant viruses to inhibit host translation, HeLa cells were infected and then were pulse-labeled with [35 S]methionine for 10 min at 4, 8, or 12 h postinfection. As in the case of RNA synthesis, the time of the pulse-label was short (10 min) compared to the turnover rates of viral and host proteins, so that labeling reflected primarily rates of synthesis. Proteins were solubilized and analyzed by SDS-PAGE and phosphorescence imaging. A representative image from analysis at 8 h postinfection is shown in Fig. 7A. All of the viruses containing wt M proteins (wtO, rwt, and rHR-M) effectively inhibited host protein synthesis compared to the mock-infected control. This can be clearly seen in regions of the gel that are devoid of viral proteins, such as the region between the L and G proteins. In contrast, the viruses containing the M51R M protein mutation (α O82, rM51R-M, and r1026-M) were much less effective in their ability to inhibit host protein synthesis. It is also apparent from Fig. 7A that the viruses containing wt M protein synthesized viral proteins at a very high level, despite the inhibition of host protein synthesis. The M protein mutants synthesized viral proteins at levels at least as high as their corresponding wt controls.

Host protein synthesis in infected cells at 4, 8, and 12 h postinfection was determined from images similar to Fig. 7A by quantitation of the radioactivity in two regions of the gel that were devoid of viral proteins (between L and G and between P and M) and is shown in Fig. 7B as the percentage of a mock-infected control. Each of the viruses containing wt M proteins (wtO, rwt, and rHR-M) effectively inhibited host protein synthesis, so that by 12 h postinfection host protein synthesis levels were 5 to 10% of the mock-infected control. In contrast, the M51R M protein mutant viruses (α O82, rM51R-M, and r1026-M) were less effective than their wt controls at repressing host translation, which was maintained at a level of 40 to 50% of control throughout the 12-h time course of the experiments. The viruses containing the TP2 and TP3 M proteins were even more defective in their ability to inhibit host protein synthesis, which was maintained at a level of 60 to 80% of control. These data together with the data in Fig. 6 demonstrate that the ability of the mutant M protein viruses to

induce IFN gene expression is correlated with a reduction in their capacity to shut off both host transcription and host translation.

Ability of recombinant viruses to synthesize viral proteins and produce infectious progeny. The rates of synthesis of viral proteins in cells infected with VSVs containing wt or mutant M proteins were determined from images similar to those in Fig. 7A. Radioactivity in the N protein band is shown in Fig. 7C and is expressed as a percentage of the N protein synthesis at 4 h postinfection with the wtO virus, which was near the maximum amount. Cells infected with recombinant viruses containing wt M protein (rwt and rHR-M viruses) synthesized N protein at different rates throughout the 12-h time course. Synthesis of N protein by rwt virus was around 55% of the level of the wtO virus control at 4 h postinfection, while that of rHR-M virus was around 80% of control. Since these viruses are isogenic except for the strain differences in their M proteins, these results reflect the influence of M protein on viral gene expression. Similar results were obtained with analysis of M protein synthesis (data not shown). The N protein/M protein ratios in cells infected with all of the viruses were the same as the ratio for wtO virus, with the exception of cells infected with rM51R-M virus, in which the ratio of N protein to M protein was approximately 40% higher. Since this difference was not observed with other viruses containing the M51R M protein mutation (α O82 and r1026-M viruses), this provides further evidence that the virus strain from which the M protein was derived influenced viral gene expression.

We have previously shown that the M51R-M proteins display no differences in their turnover rates compared to wt-M proteins (6). Results shown in this paper also indicate that, as a general trend, the viruses containing the M51R M protein mutation synthesized viral proteins at levels similar to or greater than that of their wt counterparts (Fig. 7C). The α O82 virus synthesized viral proteins at levels similar to that of the wtO virus through 8 h postinfection. However, by 12 h, the α O82 virus expressed greater amounts of N protein than wtO did. Similarly, the rM51R-M virus expressed viral proteins at levels comparable to those of rwt at early times postinfection. However, by 8 h postinfection, rM51R-M actually expressed greater amounts of N protein than any of the other viruses. Interestingly, the r1026-M virus expressed lower amounts of viral proteins at early times postinfection, but by 8 h postinfection it expressed levels of N protein comparable to its wt counterpart (rHR-M). In contrast, the recombinant viruses containing the TP2 and TP3 M proteins synthesized less viral protein than their rHR-M control at all time points (Fig. 7C). This appeared to be due to a defect in the ability of these viruses to synthesize viral RNA (data not shown). Thus, the inability of M protein mutant viruses to inhibit host RNA and protein synthesis was accompanied by defects in virus replication in the case of the rTP2-M and rTP3-M viruses, but not in the case of the viruses containing the M51R M protein mutation. The defect in viral RNA and protein synthesis exhibited by the rTP2-M and rTP3-M viruses is not dependent on IFN production, since similar results were obtained in BHK cells, which are unresponsive to IFN (data not shown). However, it is possible that a more rapid turnover of these mutant M proteins could be partly responsible for their replication defects.

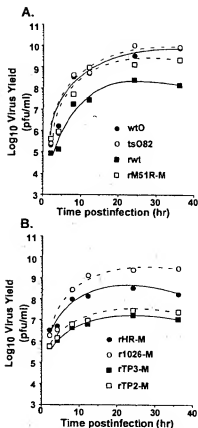


FIG. 8. Single-cycle growth analysis. HeLa cells were infected with viruses containing wt or mutant M proteins at a multiplicity of 20 PFU/cell. At 1 h postinfection, the medium was removed, and cells were washed twice. Fresh medium was added to the infected cells, and a small aliquot of the supernatant was removed at the indicated times postinfection to determine the amount of progeny virus by plaque assay. Data are the average of two independent experiments.

Single-cycle growth experiments were done to determine the ability of wt and mutant M protein viruses to produce infectious progeny (Fig. 8). HeLa cells were infected with wt and mutant viruses at a high multiplicity of infection (10 PFU/cell). At the indicated times postinfection, supernatants were collected and viral titers were determined by plaque assay on BHK cells. The α O82 virus (Fig. 8A) grew to titers as high as wtO virus. These data are similar to previous results showing that α O82 virus is not temperature sensitive for virus growth in HeLa cells (22). The rM51R-M (Fig. 8A) and r1026-M (Fig. 8B) mutant M protein viruses actually produced higher levels of infectious progeny than their wt counterparts rwt and rHR-M, respectively. These higher yields of infectious progeny also correlated with higher levels of viral protein synthesis at late times postinfection in the case of cells infected with the rM51R-M virus, as shown in Fig. 7C. Therefore, the data in Fig. 7 and 8 indicate that viruses containing the M51R M protein mutations are not defective in their ability to produce viral proteins or infectious viral progeny. This result suggests that the IFN induced by these viruses has little if any ability to inhibit virus replication in a single-cycle growth experiment.

This is the expected result, since most of the viral replicative cycle occurs before the IFN can be produced and then induce the antiviral state.

In contrast to results obtained by the M51R M protein mutants, recombinant viruses containing the TP2 and TP3 mutations grew to lower titers in single-cycle growth experiments (Fig. 8B) than did rHR-M (Fig. 8B). The lower yield of infectious progeny produced in cells infected with rTP2-M and rTP3-M viruses also correlated with reduced rates of viral RNA and protein synthesis. Therefore, the inability of these mutant M protein viruses to shut off host RNA and protein synthesis may be due in part to defects in their ability to replicate (Fig. 7) and produce infectious progeny (Fig. 8B) in infected cells.

DISCUSSION

The data presented here show that M protein plays a major role in the inhibition of IFN gene expression in VSV-infected cells. Furthermore, the ability of M protein to inhibit IFN production is genetically correlated with the overall inhibition of host RNA and protein synthesis. Previous data had shown that four IFN-inducing mutants of VSV under consideration here, α O82, T1026R1, TP2, and TP3 viruses, have mutations in their M proteins (11, 13, 16). However, it was not known whether these viruses have additional mutations in genes other than their M genes that account for their IFN-inducing phenotype. It had been shown previously that the M51R mutation in the M proteins of α O82 and T1026R1 viruses render these M proteins defective in their ability to inhibit host gene expression in the absence of other viral components (1, 6, 16, 37). These results were extended here to show that the mutant M proteins of TP2 and TP3 viruses are also defective in the inhibition of host gene expression (Fig. 1), including expression from the IFN- β promoter (Fig. 2). Thus, all of the M proteins of the IFN-inducing mutant viruses tested in this study are defective in their ability to inhibit host gene expression.

The fact that M protein inhibits expression of luciferase from a plasmid containing the IFN- β promoter in transfected cells (15) (Fig. 2) still left open the possibility that M protein is not responsible for suppression of IFN induction in virus-infected cells. For example, if the inhibition of host gene expression by M protein were to occur only at late times postinfection, as has been proposed (40), this inhibition may be too late to prevent IFN synthesis. Indeed, the argument has been made that the IFN-inducing activity of these viruses is due entirely to mutations in genes other than the M gene (34). This issue was addressed here by incorporating the M protein mutations onto the wt background of a VSV infectious cDNA clone. The resulting recombinant mutant M protein viruses induced expression of a luciferase reporter gene driven by the IFN- β promoter (Fig. 4) and induced IFN activity as measured by an IFN bioassay (Fig. 5), while the recombinant viruses containing wt M proteins did not. Furthermore, the recombinant mutant M protein viruses were defective in the inhibition of host RNA and protein synthesis (Fig. 6 and 7). These data indicate that M protein plays a main role in the inhibition of host gene expression in VSV-infected cells and is a major suppressor of IFN gene expression.

The data presented in Fig. 4 and 5 serve to establish that the

M protein mutations in the original IFN-inducing viruses account at least in part for their IFN-inducing phenotypes. However, these results do not rule out the possibility that these viruses contain additional mutations that contribute to their ability to induce IFNs. Indeed, there are several examples of IFN-inducing mutants of VSV that do not contain M protein mutations (13, 19, 34), indicating that other viral genes also play a role in determining the extent of IFN gene activation in virus-infected cells. We propose that these mutations enhance the activity of viral inducers of IFN to the extent that they overcome the inhibitory effects of M protein. Alternatively, these mutations may affect other inhibitors of IFN gene expression besides M protein.

Viruses containing the same M51R substitution in the context of M proteins derived from different virus strains (rM51R-M and r1026-M viruses) differ in their ability to induce IFN gene expression (Fig. 4 and 5). This suggests that the relative contribution of M protein versus other viral proteins in regulating IFN gene expression may be dependent on strain differences. For example, the effect of the M51R mutation may be modulated by variable surrounding amino acids in the M protein. We have also found that in different cell lines the extent of IFN induction by each of the M51R mutants also varies (unpublished data). Therefore, it is possible that the role of M protein in the activation of IFN gene expression is also dependent on the presence of specific host factors in different cell types.

The ability of mutant M protein viruses to induce IFN gene expression was correlated with defects in the ability of the mutant M proteins to inhibit host RNA and protein synthesis (Fig. 4, 5, 6, and 7). These results support our model in which wt M protein functions as a suppressor of IFN gene expression as a result of its general ability to inhibit host RNA and protein synthesis (31). According to this model, there must be other products of virus infection, such as viral dsRNA, that activate IFN gene expression, which is then suppressed by the activity of wt M protein. In the case of the mutant M protein viruses, the enhanced activation of IFN gene expression compared to that of viruses with wt M proteins would be a result of the absence of this inhibitory activity. In support of this model, the IFN-suppressing activity of wt VSV is dominant over the IFN-inducing activity of the M protein mutant T1026R1 virus in mixed infections (33). Likewise, coinfection with wt VSV and heterologous IFN-inducing viruses suppresses IFN production (33). It is also possible that the induction of IFN by mutant M protein viruses may stimulate antiviral genes, including nucleoporins, that contribute to their inability to further shut off host gene expression at the level of RNA transport (15).

How does M protein suppress IFN gene expression? VSV inhibits host gene expression at multiple levels, including inhibition of host transcription, inhibition of nuclear-cytoplasmic transport of host RNA, and inhibition of host translation (31). M protein inhibits host transcription and nuclear-cytoplasmic RNA transport both in virus-infected cells and when expressed in transfected cells in the absence of other viral components (1, 5, 24, 37, 39). Inhibition at the transcriptional level has been demonstrated by nuclear runoff assays (1, 5). The inhibition of host RNA polymerase II-dependent transcription is due, at least in part, to inactivation of the general transcription initiation factor TFIID, which is the transcription factor that binds

to the TATA box upstream of most RNA polymerase II-dependent promoters (44, 45).

The M protein-induced inhibition of host nuclear-cytoplasmic RNA transport has been attributed to the interaction of M protein with a nuclear pore component, which has been identified as the nucleoporin Nup98 (37, 39). The M protein-induced inhibition of nuclear-cytoplasmic RNA transport has been demonstrated convincingly in *Xenopus laevis* oocytes, in which there is little if any inhibition of transcription (24, 37, 39). However, it has been difficult to quantitate the contribution of the inhibition of transport in transfected mammalian cells, due to the concomitant inhibition of transcription (15, 39). The block in nuclear-cytoplasmic transport in VSV-infected cells is evident from its effects on the processing of small nuclear RNAs (20) and rRNAs (43). However, a careful series of biochemical experiments, including pulse-chase and subcellular fractionation experiments, suggested that changes in transport or turnover of host RNA in VSV-infected cells were minor compared to the profound inhibition of host transcription (41).

In addition to the inhibition of host RNA synthesis, the inhibition of host gene expression in VSV-infected cells involves a dramatic inhibition of host protein synthesis. This inhibition is not due to depletion of cellular mRNAs resulting from the inhibition of transcription (30). Instead, the inhibition is due to inactivation of host translation factors (10, 14). In contrast to the M protein-induced inhibition of host transcription and nuclear-cytoplasmic transport, M protein cannot inhibit host translation when expressed in transfected cells in the absence of other viral components (4). However, mutant M protein viruses fail to inhibit host protein synthesis as effectively as viruses containing wt M proteins (Fig. 4) (25, 27). This suggests that M protein does play a role in inhibition of host translation, but that one or more additional viral components are required to inhibit host translation. There are multiple translation initiation factors whose activity is reduced in VSV-infected cells, including eIF2, eIF4E, and eIF4B (10, 14), although it has not been determined which of these factors is inhibited in response to M protein versus other viral components.

The ability of M protein to inhibit host gene expression at multiple levels is analogous to the activity of other viral suppressors of the host IFN response, which also function at multiple levels (31). This appears to reflect the fact that no single inhibitory mechanism is completely effective at suppressing IFN production. As an example, the influenza A virus NS1 protein contains an RNA-binding domain which suppresses IFN gene activation by sequestering viral dsRNA (reviewed in reference 21). In addition, the NS1 protein contains an activation domain that enables the protein to suppress the processing and nuclear-cytoplasmic transport of host mRNAs, which appears to also play a role in suppressing the host antiviral response (reviewed in reference 31). Similarly, the vaccinia virus E3L protein suppresses activation of IFN gene expression by sequestering viral dsRNA (38). In addition, vaccinia virus encodes the K3L protein, which functions as an inhibitor of the IFN-inducible protein kinase R (12), and the B18R protein, which inhibits cellular responses to IFN by acting as a decoy receptor (3). Thus, the idea that viral proteins, such as M

protein, suppress IFN gene expression by multiple mechanisms is a common theme in virus-host interactions.

All of the mutant M proteins analyzed here were defective in their ability to inhibit host gene expression (Fig. 8). However, the viruses containing these mutations were not phenotypically identical in terms of the levels of viral protein expression or the levels of progeny virus produced. The viruses containing the M51R M protein mutation expressed viral proteins at levels at least as high as their wt controls. In fact, at late times postinfection, the α 082 and rM51R-M viruses expressed even higher levels of viral proteins than their wt counterparts (Fig. 7), leading to correspondingly higher levels of progeny virus production as seen clearly in the case of the rM51R-M virus (Fig. 8). This result indicates that the IFN induced by these viruses has little if any ability to inhibit virus replication in a single-cycle growth experiment. However, the effects of these mutations on the multiple cycles of virus infection that occur in intact animals may be quite profound, since the IFN response is a major determinant of viral pathogenesis and tissue tropism *in vivo* (reviewed in references 21 and 31). This issue will be addressed in our future experiments.

In contrast to viruses containing the M51R mutation, the viruses containing the TP2 and TP3 M protein mutations expressed lower levels of viral proteins than their control virus containing wt M protein (rHR-M virus), and they produced correspondingly lower levels of viral progeny. These data indicate that M protein mutations affect the level of viral gene expression as well as the level of host gene expression. This idea has been put forth previously, based on the observation that temperature-sensitive (ts) mutations in M protein increase the level of viral mRNA synthesis at the nonpermissive temperature (9). However, in contrast to ts M protein mutations, the TP2 and TP3 M protein mutations appear to decrease the level of viral RNA synthesis (unpublished data).

A role for M protein as a regulator of viral gene expression is also supported by the effects of M proteins from different virus strains. The rM51R and r1026-M viruses are identical except for differences in the virus strains from which their M proteins were derived. Nevertheless, viral protein expression in cells infected with the rM51R virus is approximately 40% greater at 8 to 12 h postinfection than in cells infected with the r1026-M virus (Fig. 7), providing further evidence that M protein can also regulate viral gene expression. The strain differences in their M proteins also account for the fact that r1026-M virus induced higher levels of IFN gene expression than rM51R-M virus (Fig. 4 and 5).

The results presented here support the idea that M protein inhibits IFN gene expression in parallel with the general inhibition of host RNA and protein synthesis. However, it is also possible that M protein inhibits additional steps in the production of IFNs upstream of the general transcription factor TFIID. For example, the activation of NF- κ B, one of the factors required for IFN gene transcription, is delayed in cells infected with wt VSV compared to cells infected with the TI026R1 mutant (8). This suggests that the wt M protein delays activation of NF- κ B, which could play a role in the suppression of IFN gene expression. Future experiments will determine the effect of M protein on upstream activators of IFN gene expression.

Original Research Article

Vesicular Stomatitis Virus (VSV) Therapy of Tumors

Siddharth Balachandran and Glen N. Barber

Department of Microbiology and Immunology and Sylvester Comprehensive Cancer Center,
University of Miami School of Medicine, Miami, FL 33136, U.S.A.

Summary

Vesicular stomatitis virus (VSV) is an essentially nonpathogenic negative-stranded RNA virus, the replication of which is extremely sensitive to the antiviral effects of interferon (IFN). We demonstrate here that VSV selectively induces the cytolysis of numerous transformed human cell lines in vitro, with all the morphological characteristics of apoptotic cell death. Importantly, VSV can also potently inhibit the growth of p53-null C6 glioblastoma tumors in vivo without infecting and replicating in normal tissue. With our previous findings demonstrating that primary cells containing the double-stranded RNA-activated protein kinase PKR and a functional IFN system are not permissive to VSV replication, these results suggest that signaling by IFN may be defective in many malignancies. Thus VSV might be useful in novel therapeutic strategies for targeting neoplastic disease.

JUBMB *Life*, 50: 135–138, 2000

Keywords Interferon; oncolytic virus; PKR; tumor, VSV.

INTRODUCTION

Vesicular stomatitis virus (VSV),¹ the prototypic member of the family Rhabdoviridae, is an enveloped virus with a negative-stranded RNA genome that causes a self-limiting disease in livestock and is essentially nonpathogenic in humans (1). Its simple genetic composition, the fact that it encodes five gene products, and its ability to grow to high titers in most tissue culture cell lines have made it one of the most extensively characterized of all RNA viruses. These studies have made clear that, although most tissue culture cell lines appear permissive to VSV, the virus is extremely sensitive to the antiviral actions of the interferons

(IFNs), a family of cytokines produced in response to infection, which act by inducing the expression of >30 cellular genes (1–6). The importance of IFN in innate immunity to VSV infection has been demonstrated in studies with mice rendered defective in type I IFN signaling. For example, mice lacking functional *IFNARI* or *STAT1* genes are remarkably susceptible to lethal infection by VSV, as well as by many other types of virus (3–5). Despite these advances in our understanding of the importance of IFN in host defense, the critical IFN-induced genes responsible for inhibiting replication of virus, including VSV, remain largely unknown.

Recent data from our laboratory, however, have demonstrated that embryonic fibroblasts and mice lacking the IFN-inducible double-stranded RNA-dependent protein kinase, PKR, are extremely susceptible to VSV infection, confirming that this kinase is an essential and nonredundant component of antiviral host defense (2). That VSV is capable of replicating in a majority of mammalian cell lines, but not in primary cells unless PKR function or IFN signaling is defective, implies that critical host defense mechanisms required to prevent VSV replication are impaired in cells permissive to this virus, which includes nearly all immortalized and malignant cells.

In this study, we show that several human cancer cell lines undergo rapid cytolysis when infected by VSV. Pretreating the majority of these cells with IFN only partially protects them from VSV replication and cytolysis. We also show that VSV selectively and potently inhibits the growth of highly malignant p53-defective rat C6 glioblastoma tumors in a nude mouse model. These studies indicate that VSV could provide an attractive and effective therapy against malignant disease, especially when considering the nonpathogenic nature and genetic malleability of this well-characterized virus.

EXPERIMENTAL PROCEDURES

Cell Lines, Virus, and Reagents. The BC-1 cell line was a kind gift from Dr. William Harrington (University of Miami, Miami, FL). All other cell lines were obtained from the American

Received : accepted 4 August 2000.

Address correspondence to Glen N. Barber, Ph.D., 514 Papanicolaou Bldg., 1550 NW 10th Ave., University of Miami School of Medicine, Miami, FL 33136. E-mail: gbarber@med.miami.edu

¹Abbreviations: FCS, fetal calf serum; IFN, interferon; pfu, plaque-forming units; PKR, double-stranded RNA-activated protein kinase; TUNEL, terminal deoxynucleotidyl transferase-mediated fluorescein isothiocyanate-dUTP nick-end labeling; VSV, vesicular stomatitis virus.

Type Culture Collection (Manassas, VA). HL 60 and K562 cell lines were maintained in RPMI supplemented with 10% fetal calf serum (FCS), and the BC-1 cell line was maintained in Iscove's modified Dulbecco's medium/10%FCS. All other cell lines were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% FCS. Plaque-purified VSV (Indiana strain) was used in all experiments. Viral titers were determined by standard plaque assay of serially diluted virus samples on BHK-21 cells. Except where mentioned, all other reagents were obtained from Sigma Chemicals (St. Louis, MO).

Viral Infections and Cell Viability Analyses. Plaque-purified VSV was used to infect cells in serum-free medium for 30 min at 37 °C (2). After virus absorption, cells were washed twice in phosphate-buffered saline and subsequently incubated in complete medium for the indicated times. Viability was determined by the cells' ability to exclude Trypan blue dye. Apoptosis was determined by terminal deoxynucleotidyl transferase-mediated fluorescein isothiocyanate-dUTP nick-end labeling (TUNEL) with the In Situ Cell Death Detection Kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions.

Mice and Tumor Studies. Four- to 6-week-old athymic female nu/nu mice were obtained from Jackson Laboratories (Bar Harbor, ME) and maintained in a specific pathogen-free environment. To induce tumor formation, we injected 2×10^6 C6 glioblastoma cells subcutaneously. After palpable tumors (~25 mm²) had formed, cohorts of five mice each were injected intratumorally with serial doses of VSV, 2×10^7 plaque-forming units (pfu)/dose, 4 days apart. Control mice were injected intratumorally with heat-inactivated VSV. When the tumor burdens of the control animals became excessive, all the mice were killed, and the explanted tumors were either propagated in tissue culture or cryofrozen for determination of viral titers and histopathological analyses. Organs from these mice were also examined for the presence of virus by standard plaque assay.

RESULTS AND DISCUSSION

We previously observed that embryonic fibroblasts lacking PKR, but not wild-type fibroblasts containing the kinase, were susceptible to VSV infection and viral-mediated apoptosis (2). VSV is also known to replicate in a wide variety of malignant cells and does not appear to cause important disease in humans (1). We therefore speculated that mechanisms of host defense involving PKR might be defective in cells permissive to VSV.

To further examine the ability of VSV to induce cell death in other transformed human cell lines, including those derived from breast (MCF7), prostate (PC-3), or cervical tumors (HeLa), as well as various cells derived from hematological malignancies (HL 60, K562, Jurkat, BC-1), we infected those cells with VSV as described in Experimental Procedures. We observed that VSV efficiently replicated and induced cytolysis of every established cell line tested, including BC-1, which is positive for human herpesvirus-8 (HHV-8), overexpresses Bcl-2, and is largely

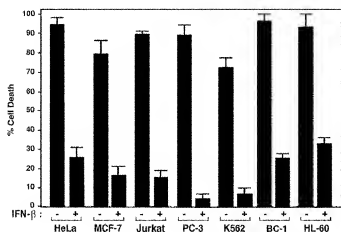


Figure 1. Several human cancer cell lines are permissive to VSV replication and lysis. MCF-7, BC-1, Jurkat, HL-60, K562, PC-3, and HeLa cells were treated with or without 1000 U/ml hIFN β (Research Diagnostics Inc., NJ) for 18 h and subsequently infected with VSV at a multiplicity of infection of 1. At 48 h post infection, viability was assessed by Trypan Blue exclusion analysis. Data represent the mean of triplicate samples \pm SD from one of two experiments with similar results.

resistant to a wide variety of apoptotic stimuli and chemotherapeutic strategies (Fig. 1) (7). The apoptotic nature of this cell death was confirmed by using TUNEL (data not shown). Supernatants were taken from infected cells and examined for VSV yield by the standard plaque assay. As shown in Table 1, all tested cell lines were remarkably permissive to VSV replication. In fact, pretreatment with 1000 U/ml human IFN β was unable to completely protect many of these cell lines from viral

Table 1

Viral yields from several human cancer cell lines infected with VSV at a multiplicity of infection of 1 (supernatants from cells treated as in Fig. 1 were analyzed for viral yield by standard plaque assay 48 h postinfection)

Cell line	hIFN β	Titer (pfu/ml)
HeLa	-	1.2×10^7
	+	8.4×10^3
MCF-7	-	1.9×10^7
	+	2.7×10^4
Jurkat	-	3.2×10^7
	+	1.3×10^3
PC-3	-	2.9×10^7
	+	1.2×10^3
K562	-	3.4×10^6
	+	4.4×10^5
BC-1	-	5.2×10^7
	+	1.2×10^5
KL-60	-	3.5×10^6
	+	2.0×10^4

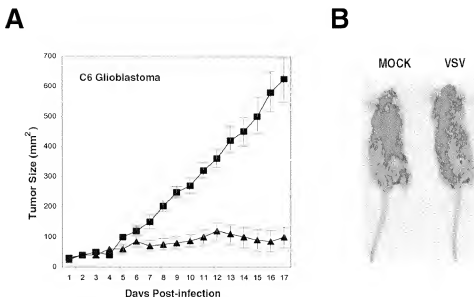


Figure 2. VSV inhibits growth of C6 glioblastoma tumors in nude mice. (A) One week after subcutaneous implantation with 2×10^6 C6 glioblastoma cells into nude mice, tumors ($n = 3$) were injected with two doses of VSV, 5×10^7 pfu/dose, 4 days apart (▲). Control tumors ($n = 3$) received equivalent amounts of heat-inactivated VSV (■). Tumor volumes were measured daily for 3 weeks. (B) Representative mice from this experiment were photographed.

replication and cytolysis, suggesting that IFN signaling may be defective in these cells.

These data indicated that VSV may be useful as an oncolytic virus in the treatment of cancer. Similar strategies have been used with viruses such as adenovirus, which has been genetically modified to preferentially replicate in p53-deficient human tumor cells (8–10). To start evaluating the use of VSV in antitumor therapy, we subcutaneously implanted athymic nude mice with 2×10^6 C6 glioblastoma cells. When palpable tumors had formed (~ 7 –14 days postinoculation, when the size of the tumors had reached ~ 0.25 mm²), the mice were infected intratumorally with VSV (2.5×10^7 pfu/ml) and monitored daily. Injection with the same amount of virus was repeated after 4 days. Administration of VSV resulted in marked repression of tumor growth in all animals tested within 17 days, when tumors in the control animals exceeded the acceptable tumor burden (Fig. 2A, B). Similar results were obtained with a single intratumoral injection of VSV (data not shown). These data highlight the potent efficacy of VSV against tumors both *in vitro* and *in vivo*.

Hematoxylin/eosin (HE)-staining of paraffin-embedded sections prepared from samples of VSV-infected C6 glioblastoma tumor tissue indicated widespread cell death; the retrieved tumors had a considerable lack of vascular infiltration and gave little evidence of tumor cell infiltration into the surrounding tissue (data not shown). Indeed, we were routinely successful in generating robust *ex vivo* growth of uninfected tumors but were unable to establish any cell lines from implanted, VSV-infected tumors (data not shown). To examine whether VSV spread beyond the virus-inoculated tumor, various tissues from the VSV-treated animals, as well as the tumors themselves, were analyzed for the

presence of residual, replicating VSV. Interestingly, examination of VSV-infected tumors for VSV 21 days after infection revealed the presence of residual virus (2×10^4 to 3.5×10^5 pfu/g) in tumor tissue derived from the C6 glioblastoma cells. However, virtually no virus (< 10 pfu/g) was detectable in the lung, brain, kidney, spleen, or liver of mice receiving VSV therapy after this period. These data show that VSV replication is restricted to tumor lineage and complements our observations confirming that no overt anomalies or sickness was apparent in the treated animals during the period of study.

Our results strongly suggest that VSV may be useful in treating human cancers. The mechanisms by which VSV is restricted to replication in tumor cells, although largely unknown, may involve disruption of key host defense mechanisms such as the IFN pathway and possibly PKR action. That PKR autophosphorylation and the phosphorylation of eukaryotic initiation factor 2 (eIF2 α) occur in VSV-permissive cell lines, however, implies that signaling pathways downstream of PKR and independent of eIF2 α are disrupted in these cells (data not shown). Alternatively, perhaps VSV can overcome the block by PKR on translation in malignant cells by a mechanism that remains to be determined. Interestingly, studies have shown that expression of activated Ras reportedly renders PKR inactive, although the mechanism by which this occurs remains unknown (11, 12). Recently, Coffey et al. (13) exploited this observation and showed that reovirus could preferentially replicate in cells transformed by Ras and might be useful as a therapy for tumors containing the activated Ras oncogene. Importantly, our data presented here, as well as results obtained by other sources, demonstrate that VSV replicates not only in those cell lines containing activated Ras

but also in tumors harboring other promoters of oncogenesis, such as overexpressed Myc, Bcl-2, or defective p53. These genetic defects occur in >90% of all known tumors, indicating that VSV could be used to treat a wide range of malignancies (14). Also noteworthy is that VSV lacks any known ability to contribute towards transformation of the cell, has a very simple genetic constitution, and is essentially nonhazardous to humans (1). Moreover, a lack of prior exposure to VSV in humans means that this treatment may not be markedly impeded by previous contact with the agent, as is the case when using other virus vectors (8–10, 13).

Indeed, the immunobiology associated with VSV infection is considerably well characterized (1). Although cytotoxic T cell responses are generally considered to be critical for resolving viral infections, substantial evidence indicates that it is the antibodies that play an indispensable role in the early phase of VSV infection. For example, B cell-deficient mice are highly susceptible to low doses of virus, although mortality can be prevented by transferring naive B cells to the mice before challenge as well as by administering immune serum after challenge (15–17). The type-specific VSV G protein is the viral antigen giving rise to the neutralizing antibodies that are critically important in eliminating VSV infection. This knowledge may be useful in designing recombinant VSV viruses to avoid host immune responses that may affect virus spread and tumor killing. The findings reported in this study have recently been complemented by Stojdl et al., who also demonstrate the potential of VSV as an oncolytic agent (18).

Another advantage of using VSV as an antitumor agent is its ability to be genetically manipulated. A family of recombinant viruses could feasibly be constructed, from which one could generate and isolate individual members presenting various G protein epitopes on their surface. In this way, rapid IgM responses that occur after 6–8 days and subsequent T cell-dependent and independent IgG responses could be avoided by administering a different viral pseudotype at repeated times until the tumor is eliminated. In addition, recombinant VSVs could be generated to express foreign proteins of choice, such as suicide cassettes or selected cytokines that enhance tumor rejection and stimulate antitumor T-lymphocyte responses.

Collectively, the data presented here show that VSV preferentially replicates and destroys immortalized or tumorigenic cells, which implies that this virus may be useful as a novel anticancer agent in the treatment of cancer.

REFERENCES

1. Wagner, R. R., and Rose, J. K. (1996) Rhabdoviridae: the viruses and their replication. In *Fields Virology*, 3rd ed. Fields, B. N., Knipe, D. M.,

- Howley, P. M., et al. (eds.). pp. 1121–1135, Lippincott–Raven Publishers, Philadelphia, PA.
2. Balachandran, S., Roberts, P. C., Brown, L. E., Truong, H., Pattnaik, A. K., Archer, D. R., and Barber, G. N. (2000) Essential role for the dsRNA-dependent protein kinase, PKR, in innate immunity to viral infection. *Immunity* **13**, 129–141.
3. Muller, U., Steinhoff, U., Reis, L. F. L., Hemmi, S., Pavlovic, J., Zinkernagel, R., and Aguet, M. (1994) Functional roles of type I and type II interferons in antiviral defense. *Science* **264**, 1918–1921.
4. Durbin, J. E., Hackenmiller, R., Simon, M. C., and Levy, D. E. (1996) Targeted disruption of the mouse *Stat1* gene results in compromised innate immunity to viral disease. *Cell* **84**, 443–450.
5. Meraz, M. A., White, J. M., Sheehan, K. C. F., Bach, E. A., Rodig, S. J., Dighe, A. S., Kaplan, D. H., Riley, J. K., Greenlund, A. C., Campbell, D., Carver-Moore, K., DuBois, R. N., Clark, R., Aguet, M., and Schreiber, R. D. (1996) Targeted disruption of the *Stat1* gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. *Cell* **84**, 431–442.
6. Stark, G. R., Kerr, I. M., Williams, B. R., Silverman, R. H., and Schreiber, R. D. (1998) How cells respond to interferons. *Annu. Rev. Biochem.* **67**, 227–264.
7. Lee, R. K., Cai, J. P., Deyev, V., Gill, P. S., Cabral, L., Wood, C., Agarwal, R. P., Xia, W., Boisse, L. H., Podack, E., and Harrington, W. J. Jr. (1999) Azidothymidine and interferon- α induce apoptosis in herpesvirus associated lymphomas. *Cancer Res.* **59**, 5514–5520.
8. Bischoff, J. R., Kim, D. H., Williams, A., Heise, C., Horn, S., Muna, M., Ng, L., Nye, J. A., Sampson-Johnnes, A., Fattae, A., McCormick, F. (1996) An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. *Science* **274**, 373–376.
9. Heise, C., Sampson-Johnnes, A., Williams, A., McCormick, F., Von Hoff, D. D., and Kim, D. H. (1997) ONYX-15, an E1B gene-attenuated adenovirus, causes tumor-specific cytolysis and antitumor efficacy that can be augmented by standard chemotherapeutic agents. *Nat. Med.* **3**, 606–608.
10. Steele, T. A. (2000) Recent developments in the virus therapy of cancer. *Proc. Soc. Exp. Biol. Med.* **223**, 118–127.
11. Mundscha, L. J., and Faller, D. V. (1992) Oncogenic Ras induces an inhibitor of the dsRNA-dependent eIF2 α kinase. *J. Biol. Chem.* **267**, 23092–23098.
12. Strong, J. E., Coffey, M. C., Tang, D., Sabinin, P., and Lee, P. W. K. (1998) The molecular basis of viral oncolysis: usurpation of the Ras signaling pathway by reovirus. *EMBO J.* **17**, 3351–3362.
13. Coffey, M. C., Strong, J. E., Forsyth, P. A., and Lee, P. W. K. (1998) Reovirus therapy of tumors with the activated Ras pathway. *Science* **282**, 1332–1334.
14. Weinberg, R. A. (1995) The molecular basis of oncogenes and tumor suppressor genes. *Ann. N. Y. Acad. Sci.* **758**, 331–338.
15. Freer, G., Burkhardt, C., Ciernik, I., Bachmann, M. F., Hengartner, H., and Zinkernagel, R. M. (1994) Vesicular stomatitis virus Indiana glycoprotein as a T cell-dependent and -independent antigen. *J. Virol.* **68**, 3650–3655.
16. Ochsenbein, A. F., Fehr, T., Lutz, C., Suter, M., Brombacher, F., Hengartner, H., and Zinkernagel, R. M. (1999) Control of early viral and bacterial distribution and disease by natural antibodies. *Science* **286**, 2156–2159.
17. Thomsen, A. R., Nansen, A., Andersen, C., Johansen, J., Marker, O., and Christensen, J. P. (1997) Cooperation of B cells and T cells is required for survival of mice infected with vesicular stomatitis virus. *Int. Immunol.* **9**, 1757–1766.
18. Stojdl, D. F., Lichty, B., Knowles, S., Marius, R., Atkins, H., Sonenberg, N., and Bell, J. C. (2000) Exploiting tumor-specific defects in the interferon pathway with a previously-unknown oncolytic virus. *Nat. Med.* **6**, 821–825.

Orthotopic models of cancer for preclinical drug evaluation: advantages and disadvantages

M.C. Bibby*

Tom Connors Cancer Research Centre, University of Bradford, Richmond Road, Bradford, West Yorkshire BD7 1DP, UK

Received 1 September 2003; accepted 17 November 2003

Abstract

Considering the enormous effort that has taken place over the years to discover new chemotherapeutic drugs for treating the common cancers, the conventional murine and xenograft test systems used to test efficacy for drug development have identified only a limited number of useful agents that are active clinically at well tolerated doses. In recent years, considerable effort has been made to develop more clinically relevant models by the use of orthotopic transplantation of tumour material in rodents. It has been shown that it is now possible to transplant tumour material from a variety of tumour types into the appropriate anatomical site and often these tumours will metastasise in a similar manner and to similar locations as the same tumour type will in human cancer. As yet, although a body of literature has amassed on the technique itself and its implications for metastasis, there are relatively few laboratories using these test systems in drug development programmes. Nevertheless, given the expertise now being developed and some interesting observations being made on the role of the tumour site on response to therapeutic agents, it is likely that the use of orthotopic systems will strengthen our ability to select the most appropriate molecules for recommended use in clinical studies.
© 2003 Elsevier Ltd. All rights reserved.

Keywords: Orthotopic; Experimental tumours; Drug discovery

1. Introduction

The selection of appropriate animal model systems in which to assess novel therapies for cancer remains controversial, but as drug discovery programmes become more geared to rational drug development, where molecules are designed to interact with a specific clinically relevant target, the selection of the appropriate *in vivo* test system is crucial to establishing the worth of such molecules. In the past, murine tumour systems were used for drug screening with mouse leukaemias being utilised as prescreens [1]. These grew very rapidly, had a high growth fraction and proved to be sensitive to a number of agents that were subsequently shown to have more activity against leukaemias and lymphomas than against solid carcinomas and sarcomas and to be toxic to the bone marrow [2]. As a result of these early screens, there is a general misconception that tumours in rodents are sensitive to drug therapy and are easy to

cure. In reality this is untrue and back in 1987 Corbett and colleagues [3] pointed out that most of the agents that had entered the clinic at that time had poor or no activity against the majority of transplantable solid tumours in mice. Modest activity is often seen, but this is usually at the expense of host toxicity [4]. In current times, new drugs are not screened for activity in panels of tumours until there are considerable mechanistic and *in vitro* data available. The National Cancer Institute (NCI) in the United States of America (USA) has had a major influence in moulding the modern strategy for drug screening ensuring that information on cytotoxicity levels and potential targets of new molecules is available at an early stage [5,6]. If a molecule has successfully passed the various tests that suggest it may have potential for further in-depth study, an appropriate *in vivo* model must be sought. This has traditionally been a panel of often poorly characterised human tumour xenografts implanted subcutaneously (s.c.) in nude mice. Although this type of model is relatively easy to operate in that it is technically straightforward and generates lots of data it is not necessarily the most

* Tel.: +44-1274-233227; fax: +44-1274-233234.

E-mail address: m.c.bibby@bradford.ac.uk (M.C. Bibby).

relevant to clinical cancer. There are publications that suggest that correlations between xenograft data and clinical activity are good [7], but there are publications that give the opposite view [8]. One major drawback of models that utilise s.c. tumour implants is that they clearly do not reproduce the primary site of the common human cancers nor do they represent the common sites of metastasis. Humans develop lung cancer in the lung, colon cancer in the colon, breast cancer in the breast etc and the common malignancies metastasise preferentially to specific sites, e.g. colon tumours metastasise to the liver. In an attempt to address these issues, orthotopic transplantation of tumour material to the appropriate anatomical site has been established. There seems to be a general view appearing amongst researchers involved in the drug discovery and development process that the use of models that more closely reflect the biological features of cancer growth and metastasis in humans will provide better prediction of potential clinical activity. This short review attempts to address some of the advantages and disadvantages of adopting this procedure for drug discovery and development.

2. Principles of orthotopic transplantation

Orthotopic transplantation of colon tumours in mice has been around for many years [9] and there is now a wealth of literature describing tumour material being implanted into most of the common sites in which cancer arises. Of course the technique not only needs to be technically feasible, but it must be ethically acceptable. It is clearly very straightforward to implant breast cancer tissue into the mammary fat pad of mice, but much more demanding on surgical skills to implant prostate cancer tissue into the prostate of a mouse for example. Early experiences were largely restricted to colon cancer and a number of general observations have been made using colon models. Orthotopic transplantation of murine colon adenocarcinoma resulted in metastatic growth in the liver [9]. At that time, the role of this type of model for new drug evaluation was not proven. Other studies since the 1950s had indicated that it was possible to grow tumours in rodents in other sites e.g. by injecting tumour cells into the left ventricle or intravenously (i.v.) [10]. Much of the effort at that time was to investigate the various stages of metastatic spread rather than to develop models for drug discovery purposes. In the case of therapeutic studies, it is important to fully characterise the model system in advance to ensure the therapeutic target is present in the model and this can be easily illustrated by a series of procedures carried out in this laboratory with a syngeneic murine model system. For this work, we utilised one of a mouse colon model panel developed in the mid-1970s [11].

These are adenocarcinomas of the colon originally induced in Naval Medical Research Institute (NMRI) mice by dimethylhydrazine. They have an advantage over some other murine systems in that they consist of a series of tumours that possess different growth characteristics, differentiation state [12] and chemosensitivity patterns to commonly used anti-cancer drugs [11]. For these early studies, we selected the MAC15 tumour. These tumours are locally invasive at the s.c. site (Fig. 1a) and previous studies had demonstrated that when a piece of this tumour was implanted intraperitoneally (i.p.) it was possible to drain ascitic fluid that contained tumour cells from these mice. These tumour cells could be grown in culture or injected i.v. when they grew in the lungs of recipient syngeneic hosts to form poorly differentiated lung colonies [13]. Using this model, it was possible to examine the influence of tumour site on response to anti-cancer drugs [14] without the necessity for intricate surgery—at first sight a very useful addition to an *in vivo* test system, although it did not address a fundamental question in colon cancer therapy viz the response of liver metastases. This needed a different approach and, in the first instance, we inoculated MAC15 cells from ascitic fluid into the wall of the caecum. This resulted in the successful growth of a poorly differentiated adenocarcinoma in the caecal wall (Fig. 1b) with metastatic deposits in the liver (Fig. 1c, d) [15]. If on the other hand, tumour pieces from the s.c. serially transplanted MAC15 were implanted into the caecal wall, a well-differentiated tumour grew at the transplantation site (Fig. 1e) and well-differentiated metastatic deposits were identified in the liver (Fig. 1f, g). Clearly, the cells recovered from the ascitic fluid did not retain the differentiation state and did not reproduce the morphology of the original tumour. Although the poorly differentiated model may well have been useful for some drug molecules e.g. standard anti-proliferative agents, it would have been less useful for evaluation of molecules that target specific stromal components of the tumour such as the tumour blood supply. As a result of the expanding literature on orthotopic transplantation and general consideration of the clinical relevance of tumour site, a number of factors that are important for drug responses have now been identified. One example is from the studies of Fidler and colleagues [16,17] who investigated the response to doxorubicin (dox) and 5-fluorouracil (5-FU) in three tumour types growing in different anatomical sites. Response to 5-FU was similar independent of site whereas only s.c. tumours responded to dox. These authors demonstrated that the difference in sensitivity to dox was probably due to overexpression of *mdr1* mRNA in the resistant sites. These experiments demonstrate the need for thorough biological investigation of the model systems employed in order to interpret the effects of therapy, an area often overlooked in preclinical drug studies.

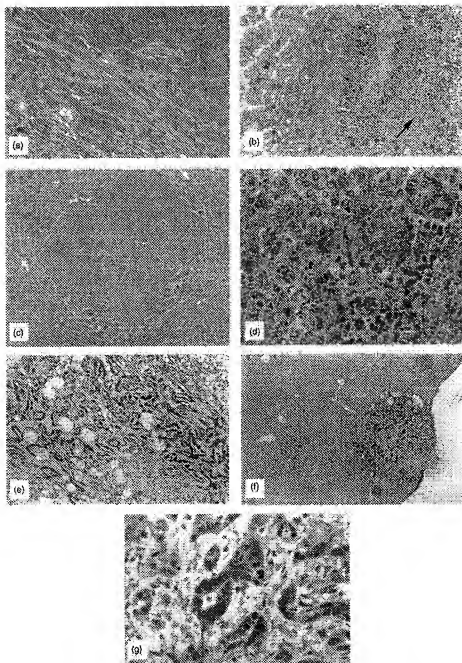


Fig. 1. Histological sections of murine colon tumours (H&E). (a) Local invasion in subcutaneously transplanted MAC15A tumour. (b) Poorly differentiated appearance of orthotopically implanted MAC15A cells in the mouse caecum. (c) Poorly differentiated colon metastasis of orthotopically implanted MAC15A cells. (d) High power of (c). (e) Well-differentiated MAC15 tumours implanted in mouse caecum. (f) Single well-differentiated metastasis of orthotopically implanted MAC15 tumours. (g) High power of (f) showing glandular appearance of metastasis. (original images b–g courtesy of Ramakrishnan, 1983 [15]). MAC15A cells were derived from ascitic fluid [13].

3. Advantages of an orthotopic system

Given the fact that many tumour types can now be grown successfully in orthotopic sites [18–21], what are the real advantages of such systems for drug discovery and development? It is now clear that the process of metastasis is more efficient in orthotopically implanted

tumours and closely mimics human metastasis [22]. There are impressive studies describing metastasis of orthotopic tumours to clinically relevant sites e.g. prostate and breast to bone [23,24]. One of the most obvious advantages then of orthotopic systems is that attempts to target processes in local invasion e.g. inhibition of proteases or interfering with the process of angiogenesis

can be carried out in a more clinically relevant site. The identification of different receptor expression in endothelium from different organs [25] clearly demonstrates the usefulness of using appropriate sites for design and investigation of novel targeted anti-angiogenesis therapies. Studies with anti-vascular drugs have been carried out in models of colon cancer. 5,6-dimethyl xanthone acetic acid (DMXAA) [26] and Combretastatin A-4 phosphate (CA-4) [27] have been shown to cause vascular shutdown and haemorrhagic necrosis in vascularised colon tumours in orthotopic and metastatic sites in mice [28,29]. Blood flow effects with CA-4 occurred well below the maximum tolerated dose (MTD) whereas DMXAA had a narrow therapeutic window. In subsequent clinical trials, CA-4 appeared to be more effective at causing changes in blood flow in human tumours. More recently, another combretastatin analogue, combretastatin A-1 phosphate, has been shown to be more effective than CA-4 at causing haemorrhagic necrosis in two models of human colon tumour liver metastasis [30]. It remains to be seen whether data from orthotopic tumour systems give a better indication of potential clinical activity of these antivascular drugs.

Orthotopic transplantation has occasionally been useful in identifying potential pitfalls of s.c. tumour models. There have been a number of publications indicating that it is possible to potentiate the activity of standard and investigational agents in experimental tumours by combination with a variety of vaso-active agents. This approach relies on the described lack of smooth muscle and innervation of neovasculature in solid tumours [31]. Many agents alter blood flow in tumours [32], but most studies utilised the anti-hypertensive hydralazine that is effective at reducing blood flow in transplantable tumours in rodents [33,34]. Hydralazine was shown to enhance the effectiveness of bioreductive drugs such as RSU1069 [35], Tirapazamine [36], EO9 [37] and mitomycin C [38] and a couple of direct-acting cytotoxic agents, melphalan [39] and taurumustine [40]. These studies were all carried out in s.c. transplanted models and although such studies indicated a potential therapeutic strategy this has not been shown to work clinically. Studies in rodents with primary malignancies [41] indicated less of an effect with hydralazine and studies from this laboratory indicated that hydralazine was more effective at shutting down blood supply to a s.c. transplanted murine colon tumour than to the same tumours transplanted orthotopically [42]. Although the approach of altering tumour blood flow for therapeutic gain in humans seems to be little studied at present, Rowell and colleagues [43] showed by the use of SPECT and ^{99m}Tc -HMPO that single dose oral hydralazine caused the blood flow through human lung tumours to increase rather than decrease. Experimental evidence suggests that orthotopically transplanted tumours may be more

appropriate models in which to investigate these physiological strategies than the usual s.c. transplanted tumour models.

To date, considering the large body of published information on orthotopic systems, few studies have been concerned with investigating the effects of chemotherapy in general and even less have been used in pre-clinical studies of novel drugs. However, some of these studies suggest that the results are likely to better reflect the activity in patients. The Hoffman group which is prolific in the area of orthotopic tumour research, using an *in vivo* model of small-cell lung cancer (SCLC), showed that cisplatin had significant effects against lung tumours, but was ineffective against the same tumours growing s.c. [44]. Mitomycin C was ineffective against the lung tumours thus reflecting the clinical situation. The authors concluded that their data suggested that tumours grown orthotopically reflect the clinical effects of drugs on human SCLC more closely than the tumours growing s.c. The Fidler group made the equally valid point that human colon xenografts growing s.c. in nude mice often respond to dox, whereas human colon cancer does not [17]. Despite these very interesting studies, there is a need for more in-depth assessment of the potential advantages of orthotopic over s.c. tumours by examining currently useful chemotherapeutic agents against common cancer types.

4. Disadvantages of orthotopic models

It is relatively easy to identify disadvantages of orthotopic transplantation, the most obvious limitation being technical skill. The procedures are generally far more difficult and time-consuming, hence more expensive than for conventional s.c. models. Endpoints for determining the effects of therapy are more complex than the normal tumour measurement in s.c. models and ensuring that animal suffering is kept to a minimum, although essential, can be difficult. As imaging studies are developed, improved and become more widely available, the endpoint becomes less of a problem and animal usage can be reduced as the ability to follow the effects of therapy sequentially in individual animals becomes possible.

Of course the major goal of drug discoverers is to develop effective therapies for metastatic deposits of the major common malignancies. Very often the surgeon has successfully removed the primary tumour, but the patient will die later of metastatic disease. In our experience, metastasis from an orthotopically transplanted colon tumour in a mouse can be a late event, at least the mouse may succumb to the primary tumour prior to significant metastasis to the liver being obvious. The identification of a suitable endpoint other than the scientifically and morally unacceptable one of survival

can be difficult. However, if the "primary" deposit is surgically excised and the mouse allowed to recover, metastatic deposits occur in the liver after a few weeks. This makes the procedures more complex, but certainly produces a good model of large bowel cancer.

The development and establishment of stable green fluorescent protein (GFP)-expressing cell lines that permit detection and allow visualisation of growth of the tumour and metastases in live tissue [45] has had a major impact on research with orthotopic models. At the time this work represented a significant improvement over the use of the *Escherichia coli* β galactosidase (*lacZ*) gene to identify metastases where it was necessary to use histological preparations of tissues [46]. A number of publications have now appeared on the use of GFP to monitor orthotopic and metastatic growth of tumours non-invasively and this technique would lend itself to preclinical drug evaluation. A non-invasive imaging technique for monitoring luciferase-expressing human prostate tumours and metastasis in nude mice after i.p. inoculation of luciferase has also been described [47] and a particularly interesting approach is the use of *in vivo* bioluminescent imaging to study bone metastasis in a model of prostate cancer [24]. Animal positron emission tomography (PET) studies have been successfully used to monitor the effects of the anti-vascular agent CA-4 in murine liver metastases [48]. Although these imaging studies have indicated the potential for monitoring tumour growth non-invasively, the techniques are not yet widely available and it is still unclear whether the use of orthotopic versus s.c. tumours results in a better prediction of clinical response. The matrix metalloproteinase inhibitor, Batimastat, was shown to reduce tumour progression in an orthotopic model of colon cancer [49], but this compound has subsequently been shown to be disappointing in clinical trials, so it appears that orthotopic tumours can still overestimate potential clinical efficacy.

5. Conclusions

In assessing the question as to whether orthotopic models are better for drug studies than the more conventional s.c. tumours, it is necessary to first think about the questions we are attempting to answer [50]. What do we need from a preclinical tumour model used in drug discovery? The most important question can be answered in a simple mouse system i.e. can effective drug concentrations based on previous *in vitro* data be achieved *in vivo* and are these concentrations tolerated without major toxicity? Assuming this to be in the affirmative, the next question to address is: Is the molecule reaching and, more importantly, interacting with its designated target? A prerequisite of any model then, whether it be a s.c. or orthotopic tumour, must be that

not only is the target present, but that it is possible in that model to demonstrate evidence of the mechanism of action i.e. a pharmacodynamic endpoint. The best approach to selecting an animal model for drug efficacy studies is to design the model to address each question. It is likely that a number of clinically relevant targets will be better represented by orthotopic tumour systems that mimic the morphology, microenvironment and growth and metastatic patterns of human cancer. Angiogenesis is one of the areas that appears appropriate for more in-depth study in this regard.

In conclusion, there have been very significant developments in orthotopic transplantation techniques for studying the process of cancer metastasis. It is clear that the appropriate microenvironment leads to the development of the metastatic phenotype and there are further opportunities to investigate the molecular events associated with cancer progression. However, there is still some way to go in determining the real advantages these techniques may have in improving our ability to design and evaluate the most effective molecules for treating human metastatic disease. The time is right to fully characterise these models to establish their real worth as predictors of therapeutic outcome of clinical disease.

Acknowledgements

MCB is supported by Cancer Research UK

References

- Plowman J, Dykes DJ, Hollingshead M, Simpson-Herren L, Alley MC. Human tumor xenograft models in NCI drug development. In Teicher B, ed. *Anticancer Drug Development Guide: Preclinical Screening, Clinical Trials and Approval*. Totowa, NJ, Humana Press, 1997, 101-125.
- Muggia FM. Closing the loop: providing feedback on drug development. *Cancer Treat Rep* 1987; 71, 1-2.
- Corbett TH, Valeriote FA, Baker LH. Is the P388 murine tumor no longer adequate as a drug discovery model? *Investigational New Drugs* 1987; 5, 3-20.
- Double JA, Bibby MC. Therapeutic Index: a vital component in selection of anticancer agents for clinical trial. *J Natl Cancer Inst* 1989; 81, 988-994.
- Boyd MR. Status of the NCI preclinical antitumor drug discovery screen. In De Vita Jr. VT, Hellman S, Rosenberg SA, eds. *Cancer Principles and Practice of Oncology Update*, vol 3. Philadelphia, Lippincott, 1989, 1-12.
- <http://dtp.nci.nih.gov>.
- Fiebig HH, Berger DP. Preclinical Phase II trials. In Boven E, Winograd B, eds. *The Nude Mouse in Oncology Research*. Boca Raton, CRC Press, 1995, 318-335.
- Johnson JL, Decker S, Zaharevitz D, et al. Relationships between drug activity in NCI preclinical *in vitro* and *in vivo* models and early clinical trials. *Br J Cancer* 2001; 84, 1424-1431.
- Tan MH, Holyoke ED, Goldrosen MC. Murine colon adenocarcinomas: syngeneic orthotopic transplantation and subsequent hepatic metastases. *J Natl Cancer Inst* 1977; 59, 1537-1544.

10. Sugarbaker EV. Patterns of metastasis in human malignancies. *Cancer Res Rev* 1981, 2, 235–278.
11. Double JA, Ball CR. Chemotherapy of transplantable adenocarcinomas of the colon in mice. *Cancer Chemother Rep* 1975, 59, 1083–1089.
12. Cowen DM, Double JA, Cowen PM. Some biologic characteristics of transplantable lines of mouse adenocarcinoma of the colon. *J Natl Cancer Inst* 1980, 64, 675–681.
13. Double JA, Cifuentes de Castro L. Chemotherapy of transplantable adenocarcinomas of the colon in mice II development and characterisation of an ascitic line. *Cancer Treat Rep* 1978, 62, 85–90.
14. Bibby MC, Double JA, Morris CM. Antitumour activity of TCNU in a panel of transplantable murine colon tumours. *Europ J Cancer Clin Oncol* 1988, 24, 1361–1364.
15. Ramakrishnan S. Establishment and characterisation of an experimental metastatic model of an adenocarcinoma of the mouse colon. MPhil thesis, University of Bradford, Bradford, UK, 1983.
16. Dong Z, Radinsky R, Fan D, Tsan R, Bucana CD, Wilmanns C, Fidler IJ. Organ-specific modulation of steady-state mdr gene expression and drug resistance in murine colon cancer cells. *J Natl Cancer Inst* 1994, 86, 913–920.
17. Fidler IJ, Wilmanns C, Staroselsky A, Radinsky JR, Dong Z, Fan D. Modulations of tumor cell response to chemotherapy by the organ environment. *Cancer Metastasis Rev* 1994, 13, 209–222.
18. Fidler IJ, Naito S, Pathak S. Orthotopic implantation is essential for the selection, growth and metastasis of human renal cell cancer in nude mice. *Cancer Metastasis Rev* 1990, 9, 149–165.
19. Fidler IJ. Orthotopic implantation of human colon carcinomas into nude mice provides a valuable model for the biology and therapy of metastasis. *Cancer Metastasis Rev* 1991, 10, 229–243.
20. Gutman M, Fidler IJ. Biology of human colon cancer metastasis. *World J Surg* 1995, 19, 226–234.
21. Hoffman RM. Fertile seed and rich soil. In Teicher B, ed. *Anticancer Drug Development Guide: Preclinical Screening, Clinical Trials and Approval*. Totowa NJ, Humana Press, 1997, 127–144.
22. Killian JJ, Radinsky R, Fidler IJ. Orthotopic models are necessary to predict therapy of transplantable tumors in mice. *Cancer Met Rev* 1999, 17, 279–284.
23. Yang M, Jiang P, Sun F-X, et al. A fluorescent orthotopic bone metastasis model of human prostate cancer. *Cancer Research* 1999, 59, 781–786.
24. Rosol TJ, Tannehill-Gregg SH, LeRoy BE, Mandl S, Contag CH. Animal models of bone metastasis. *Cancer* 2003, 97(Suppl. 3), 748–757.
25. Pasqualini R, Ruoslahti E. Organ targeting in vivo using phage display peptide libraries. *Nature* 1996, 380, 364–366.
26. Atwell GJ, Rewcastle GW, Baguley BC, Denny WA. Synthesis and antitumour activity of topologically related analogues of flavone acetic acid. *Anticancer Drug Design* 1989, 4, 161–169.
27. Pettit GR, Temple CJR, Nnanyan VL, et al. Antinoplastic agent 322. Synthesis of combretastatin A4 prodrugs. *Anticancer Drug Des* 1995, 10, 299–309.
28. Laws AL, Matthew AM, Bibby MC, Double JA. The activity of 5,6-MeXAA on a subcutaneous and orthotopic model of human colon cancer. *Br J Cancer* 1995, 71(Suppl. XXIV), 40.
29. Grosios K, Holwell SE, McGown AT, Pettit GR, Bibby MC. In vivo and in vitro evaluation of combretastatin A-4 and its sodium phosphate prodrug. *Br J Cancer* 1999, 81, 1318–1327.
30. Holwell SE, Cooper PA, Thompson MJ, et al. Anti-tumor and anti-vascular effects of the novel tubulin-binding agent combretastatin A-1 phosphate. *Anticancer Res* 2002, 22, 3933–3940.
31. Denekamp J. Endothelial cell proliferation as a novel approach to targeting tumour therapy. *Br J Cancer* 1982, 45, 136–139.
32. Hirst DG, Wood PJ. The control of tumour blood flow for therapeutic benefit. *BIR Rep* 1989, 19, 76.
33. Chan RC, Babbs CF, Vetter RJ, Lamar CH. Abnormal response of tumor vasculature to vasoactive drugs. *J Natl Cancer Inst* 1984, 72, 145–150.
34. Jirtle RL. Chemical modifications of tumour blood flow. *Int J Hyperthermia* 1988, 4, 355–371.
35. Chaplin DJ, Acker B. The effect of hyalazine on the tumor cytotoxicity of the hypoxic cell cytotoxin RSU-1069 evidence for therapeutic gain. *Int J Radiat Oncol Biol Phys* 1987, 13, 579–585.
36. Brown JM. Exploitation of bioreductive agents with vaso-active drugs. In Fielden EM, Fowler JF, Hendry JH, Scott D, eds. *Radiation Research: Proceedings of the 8th International Congress of Radiation Research*. London, Taylor & Francis, 1987, 719–724.
37. Bibby MC, Sleight NR, Loadman PM, Double JA. Potentiation of EO9 anti-tumour activity by hyalazine. *Eur J Cancer* 1993, 29A, 1033–1035.
38. Cowen SE, Loadman PM, Double JA, Bibby MC. Hyalazine alters murine mitomycin C plasma pharmacokinetics—a possible explanation of drug potentiation. *Br J Cancer* 1994, 69(Suppl XXI), 41.
39. Stratford IJ, Adams GE, Godden J, Nolan J, Howells N, Timpson N. Potentiation of the anti-tumour effect of melphalan by the vasoactive agent hyalazine. *Br J Cancer* 1988, 58, 122–127.
40. Quinn PKM, Bibby MC, Cox JA, Crawford SM. The influence of hyalazine on the vasculature, blood perfusion and chemosensitivity of MAC tumours. *Br J Cancer* 1992, 66, 323–330.
41. Field SB, Needham S, Burney JA, Maxwell RJ, Coggle JE, Griffiths JR. Differences in vascular responses between primary and transplanted tumours. *Br J Cancer* 1991, 63, 723–726.
42. Cowen SE, Bibby MC, Double JA. Characterisation of the vasculature within a murine adenocarcinoma growing in different sites to evaluate the potential of vascular therapies. *Acta Oncol* 1995, 43, 357–360.
43. Rowell NP, Flower MA, McCready VR, Cronin B, Horwich A. The effects of single-dose oral hyalazine on blood flow through human lung tumours. *Radiother Oncol* 1990, 18, 283.
44. Kuo T-H, Kubota T, Watanabe M, et al. Site-specific chemosensitivity of human small-cell lung carcinoma growing orthotopically compared to subcutaneously in SCID mice: the importance of orthotopic models to obtain relevant drug evaluation data. *Anticancer Res* 1993, 13, 627–630.
45. Chishima T, Miyagi Y, Wang X, et al. Cancer invasion and micrometastasis visualized in live tissue by green fluorescent protein expression. *Cancer Res* 1997, 57, 2042–2047.
46. Lin WC, Pretlow TP, Pretlow TG, Culp LA. Bacterial lacZ gene as a highly sensitive marker to detect micrometastasis formation during tumor progression. *Cancer Res* 1990, 50, 2808–2817.
47. El Hilali N, Rubio N, Martínez-Villacampa M, Blanco J. Combined non-invasive imaging and luminometric quantification of luciferase-labeled human prostate tumors and metastases. *Laboratory Investigation* 2002, 82, 1563–1571.
48. Zhao S, Moore JV, Waller ML, et al. Positron emission tomography of murine liver metastases and the effects of treatment by combretastatin A-4. *Eur J Nuclear Medicine* 1999, 26, 231–238.
49. Wang X, Fu X, Brown PD, Crimmin BJ, Hoffman RM. Matrix metalloproteinase inhibitor BB-94 (Batimastat) inhibits human colon tumor growth and spread in a patient-like orthotopic model in nude mice. *Cancer Research* 1994, 54, 4726–4728.
50. Bibby MC. Making the most of rodent tumour systems in cancer drug discovery. *Br J Cancer* 1999, 79, 1633–1640.

ORIGINAL ARTICLE

Oncolytic activity of vesicular stomatitis virus in primary adult T-cell leukemia

R Césaire^{1,8}, S Olié^{2,3,8}, E Sharif-Askari^{2,3,8}, M Loignon^{2,3}, A Lézin¹, S Olindo⁴, G Pangelatti⁵, M Kazanjji⁶, R Aloyz^{2,3}, L Panasci^{2,3}, JC Bell⁷ and J Hiscott^{2,3}

¹Laboratoire de Virologie-Immunologie et UMR433 INSERM, Centre Hospitalier Universitaire de Fort-de-France, Martinique, France; ²Molecular Oncology Group, Lady Davis Institute for Medical Research, Jewish General Hospital, Montreal, Quebec, Canada; ³Departments of Microbiology and Medicine, McGill University, Montreal, Canada; ⁴Service de Neurologie, Centre Hospitalier Universitaire de Fort-de-France, Martinique, France; ⁵Service de Médecine Interne, Centre Hospitalier Universitaire de Fort-de-France, Martinique, France; ⁶Laboratoire Rétrovirus, Institut Pasteur de Cayenne, French Guyana, France and ⁷Ottawa Regional Cancer Centre, University of Ottawa, Ottawa, Canada

Treatments for hematological malignancies have improved considerably over the past decade, but the growing therapeutic arsenal has not benefited adult T-cell leukemia (ATL) patients. Oncolytic viruses such as vesicular stomatitis virus (VSV) have recently emerged as a potential treatment of solid tumors and leukemias *in vitro* and *in vivo*. In the current study, we investigated the ability of VSV to lyse primary human T-lymphotropic virus type 1 (HTLV-1)-infected T-lymphocytes from patients with ATL. *Ex vivo* primary ATL cells were permissive for VSV and underwent rapid oncolysis in a time-dependent manner. Importantly, VSV infection showed neither viral replication nor oncolysis in HTLV-1-infected, nonleukemic cells from patients with HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), and in naive CD4+ T-lymphocytes from normal individuals or in *ex vivo* cell samples from patients with chronic lymphocytic leukemia (CLL). Interestingly, activation of primary CD4+ T-lymphocytes with anti-CD3/CD28 monoclonal antibody, and specifically with anti-CD3, was sufficient to induce limited viral replication and oncolysis. However, at a similar level of T-cell activation, VSV replication was increased fourfold in ATL cells compared to activated CD4+ T-lymphocytes, emphasizing the concept that VSV targets genetic defects unique to tumor cells to facilitate its replication. In conclusion, our findings provide the first essential information for the development of a VSV-based treatment for ATL.

Oncogene (2006) 25, 349–358. doi:10.1038/sj.onc.1209055; published online 26 September 2005

Keywords: ATL; HTLV-1; HAM/TSP; VSV; apoptosis; oncolysis

Introduction

Adult T-cell leukemia (ATL) is an aggressive malignancy of mature activated, CD4/CD25+ T-lymphocytes that occurs worldwide in populations where human T-lymphotropic virus type 1 (HTLV-1) infection is endemic – southwestern Japan, the Caribbean Basin, intertropical Africa, and the southern United States. HTLV-1 has also been etiologically linked to a neurological disorder, HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Uchiyama *et al.*, 1977; Hinuma *et al.*, 1982; Gessain *et al.*, 1985; Osame *et al.*, 1986). Mechanistically, the HTLV-1 Tax oncoprotein plays a central role in the initiation of transformation by transactivating several cellular pathways (Yoshida, 2001; Franchini *et al.*, 2003), and by promoting the oligo/polyclonal expansion of HTLV-1-infected T cells (Mortreux *et al.*, 2003). Subsequently, Tax-mediated accumulation of host genome alterations leads to Tax-independent proliferation, escape from the CD8+ T-lymphocyte anti-Tax response, and emergence of the malignant CD4/CD25+ T-lymphocyte clone (Yasunaga and Matsuoka, 2003).

Despite extensive progress in the understanding of the molecular virology and immunology of HTLV-1-associated diseases, there has been only minimal improvement in the treatment of ATL. Conventional as well as high-dose chemotherapy has shown only limited benefit, and the aggressive subtypes of ATL carry a very poor prognosis, with a median survival of less than 1 year (Shimoyama, 1991). The combination of antiviral nucleosides and interferon (IFN)- α has been shown to improve the response rate and survival; however, most patients relapse and succumb to disease, thus underlining the need for new therapeutic approaches (Bazarbachi *et al.*, 2004).

One promising and novel cancer therapeutic approach against tumor cells is virotherapy. Several naturally occurring or genetically engineered viruses are effective in killing tumor cells in many experimental models (Steele, 2000; Wu *et al.*, 2001; Chiocca, 2002). Recently, vesicular stomatitis virus (VSV), an enveloped

Correspondence: Dr J Hiscott, Molecular Oncology Group, Lady Davis Institute for Medical Research, Jewish General Hospital, Room 526, 3755 Cote-Ste. Catherine Road, Montreal, Quebec, Canada H3T 1E2. E-mail: john.hiscott@mcgill.ca

⁸These authors contributed equally to this work.

Received 19 April 2005; revised 12 July 2005; accepted 27 July 2005; published online 26 September 2005

negative-strand RNA virus belonging to the *Rhabdoviridae* family, has been shown to possess intrinsic oncolytic properties (Balachandran and Barber, 2000; Stojdl et al., 2000). VSV can infect a wide variety of mammalian cells and host defense to VSV infection is based on the antiviral activity of IFN before the generation of neutralizing antibody response (Grigera et al., 1996). The ability of VSV to replicate in and lyse tumor cells occurs in part due to defects in the IFN antiviral response in tumor cells (Stojdl et al., 2000, 2003; Fernandez et al., 2002). Recently, Stojdl et al. (2003) demonstrated that 80% of VSV-permissive cell lines had impaired responses to either IFN- α or IFN- β . Complete destruction of cell line-derived xenograft tumors in animal models following intratumoral injection of wild-type or genetically modified VSV have been documented (Balachandran and Barber, 2000; Stojdl et al., 2000, 2003; Balachandran et al., 2001; Fernandez et al., 2002; Ebert et al., 2003; Huang et al., 2003; Obuchi et al., 2003). Furthermore, systemic intravenous administration of VSV in mouse models has also shown potency in treating primary and metastatic tumors (Balachandran et al., 2001).

The importance of IFN in the host defense against VSV was highlighted by demonstrating that mice harboring defects in IFN signaling are highly sensitive to normally sublethal exposure to VSV (Muller et al., 1994; Durbin et al., 1996), a situation that is dramatically reversed by prophylactic IFN treatment (Stojdl et al., 2003). Recent analysis has demonstrated that defective control of mRNA translation initiation plays a crucial role in cell permissiveness to VSV (Balachandran and Barber, 2004), and that translation control downstream of PKR activation, frequently dysregulated in many transformed cells, can cooperate with attenuated IFN antiviral activity to facilitate VSV oncolysis.

While most studies of VSV oncolysis *in vitro* and/or in animal models have been performed with cell lines, preclinical data in primary cancer cells are still lacking. In this study, we investigated the oncolytic activity of VSV in primary CD4⁺ T-lymphocytes from ATL patients. *Ex vivo* VSV infection induced significant oncolytic activity in primary ATL cell in a time- and dose-dependent manner. These studies provide the first essential information for the development of a VSV-based treatment for ATL.

Materials and methods

Cells and reagents

HTLV-1 T-cell lines MT-2 and MT-4 cells were purchased from American Type Culture Collection (ATCC) and maintained in RPMI 1640 medium supplemented with 10% FCS (Wisent) and 100 U/ml penicillin/streptomycin (referred to as complete RPMI 1640). The human Epstein-Barr virus-transformed B-cell chronic lymphocytic leukemia (B-CLL) I-83 cell line and Epstein-Barr virus-negative B-CLL WSU cell line were cultured in complete RPMI 1640. Peripheral blood mononuclear cells (PBMCs) from four ATL patients (samples ATL-1, ATL-2, ATL-3, and ATL-4), two patients with HAM/

TSP (HAM-1 and HAM-2), three patients with B-CLL (CLL-1, CLL-2, and CLL-3), one patient with T-CLL (CLL-4), and three healthy individuals were obtained after informed consent. PBMCs were isolated by centrifugation (400 g at 20°C for 25 min) of blood on a Ficoll-Hypaque gradient (Amersham Pharmacia Biotech). PBMCs were resuspended in RPMI 1640 medium supplemented with 15% heat-inactivated FBS, 100 U/ml penicillin-streptomycin, and 70 U/ml of human-recombinant IL-2 (rIL-2, Roche Diagnostics, Mannheim, Germany).

Isolation of T cells

Freshly isolated PBMCs were incubated with CD4 enrichment cocktail (Miltenyi Biotec) and CD4⁺ T-lymphocytes were negatively selected with the high-speed autoMACS system (Miltenyi Biotec) according to the manufacturer's instructions. In all cases, the purity of CD4⁺ T-lymphocytes was between 90 and 95% as determined by flow cytometry. Isolated CD4⁺ T cells were cultured in complete RPMI 1640.

T-cell activation

Purified CD4⁺ T-lymphocytes (10⁶/ml) were mock activated or activated *in vitro* for 24 h with 5 μ g/ml of immobilized anti-CD3 monoclonal antibody, and 1 μ g/ml of immobilized anti-CD28 monoclonal antibody (BD Biosciences), in the presence of 70 U/ml of rIL-2. Cells were then harvested, washed, and cultured in complete RPMI 1640.

Virus production, quantification, and infection

Wild-type VSV (Indiana serotype) and rVSV-GFP were propagated in BHK-21 cells as described previously (Stojdl et al., 2003). Viruses were obtained from cell-free supernatants and titrated on Vero cells by standard plaque assay. Cells were mock infected or infected with VSV at an MOI of 1 PFU/cell for 1 h in RPMI 1640 at 37°C. In some experiments, caspase-3 inhibitors zVAD-FMK or D-VAD-FMK were added to a final concentration of 100 μ M at 20 min prior to VSV infection. The cells were then washed with PBS and incubated with complete RPMI 1640 at 37°C for the indicated times. Following incubation, cells were harvested in PBS, pelleted, and processed for protein analysis. For indicated experiments, cells were mock infected or infected with rVSV-GFP under similar conditions and GFP expression was analysed by FACScaliburTM flow cytometry.

Immunoblot analysis

Cells destined for immunoblotting were washed with PBS and lysed in lysis buffer (0.05% NP40, 1% glycerol, 30 mM NaF, 40 mM β -glycerophosphate, 10 mM Na₂VO₄, 10 ng/ml of protease inhibitors (leupeptine, aprotinin, and pepstatin)). Protein concentration was determined with Bio-Rad protein assay reagent (BioRad), and 20 μ g of protein was then resolved using 12% SDS-PAGE and transferred to nitrocellulose membrane (Hybond C Super; Amersham, Oakville, Canada). Blots were blocked for 1 h at 25°C in 5% nonfat dried milk in PBST (PBS + 0.5% Tween-20). Membranes were then incubated overnight with rabbit anti-VSV, anti-Rho GDP-dissociation inhibitor (Rho-GDI) (BD Pharmingen, 1:5000), anti-procaspase-3 (Upstate, 1:1000), anti-cleaved caspase-3 (Cell Signaling, 1:2000), anti-DNA fragmentation factor-45 (DFF-45) (Shari-Askari et al., 2001) (1:3500), or mouse anti- β -actin (Chemicon, 1:10000) at 25°C. The blots were then washed three times in PBST and reincubated in the presence of horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibodies (Amersham, 1:3000) for 1 h at 25°C.

Following three washes with PBST, the proteins were detected with the enhanced chemiluminescence (ECL) kit (Amersham, Oakville, Canada).

Immunofluorescence staining and analysis

After washing twice with PBS, cells were stained with monoclonal allophycocyanin-labeled anti-CD4, phycoerythrin (PE)-labeled anti-CD25 or PE-labeled anti-CD69 (BD Pharmingen), for 30 min in PBS/1% FCS. After a final wash with ice-cold PBS, cells were resuspended in 400 μ l FACS[®] buffer (PBS/CytoFix (BD Pharmingen)). Flow cytometric analyses (1×10^4 cells/measurement) were performed by FACScalibur[™] flow cytometer with CELLQuest[™] software (Becton Dickinson).

Measurement of apoptosis

Apoptotic cells were analysed by annexin V/7-amino-actinomycin D (7-AAD) double staining for detection of the apoptotic plasma membrane (phosphatidylserine translocation). Mock- or VSV-infected cells were washed and resuspended in 300 μ l of ice-cold annexin V binding buffer (HEPES-NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂ (BD Pharmingen)), incubated on ice for 15 min with 1 μ l of each of fluorescein isothiocyanate (FITC)-conjugated annexin V and 7-AAD (BD Pharmingen), and followed by flow cytometric analysis. For DNA content staining, cells were collected, washed in ice-cold PBS/5 mM EDTA, and fixed in ethanol (70% in PBS) overnight at -20°C . After fixation, cells were then washed in PBS, and stained with a PI staining solution (PBS, 50 μ g/ml PI, 100 U/ml RNase A and 1 mg/ml glucose) for 2 h at room temperature before FACS[®] analysis. For each sample, the forward versus right-angle scatter cytogram was used to exclude debris and aggregates. Apoptosis was determined by quantification of the sub-G₀ population (1×10^4 cells/measurement).

Results

VSV replicates and induces rapid cell death in HTLV-1-transformed cells

Wild-type VSV has been shown to induce potent cytolytic effects against a wide range of tumor cells, including 80% of the NCI human tumor cell line panel (Weinstein *et al.*, 1997). Thus, we first sought to determine the ability of VSV to replicate and induce cell death in the HTLV-1-infected T-cell lines, MT-2 and MT-4. VSV infection was accompanied by cell death; quantification by acridine orange-ethidium bromide fluorescent-dye staining showed that cell death was time dependent (Figure 1a), and by 48 h post-VSV infection, no significant viable cells were detected. Cell death was correlated with VSV replication; as shown in Figure 1b, after infection with a VSV strain expressing GFP (rVSV-GFP), a significant percentage of cells were positive for GFP expression in both MT-2 and MT-4, illustrating the permissiveness of HTLV-1-transformed T cells for VSV infection. At 12 h postinfection, 45% of infected cells were GFP positive in MT-4 cells as compared to 10% in MT-2 cells, suggesting higher VSV replication in MT-4 than MT-2 cells.

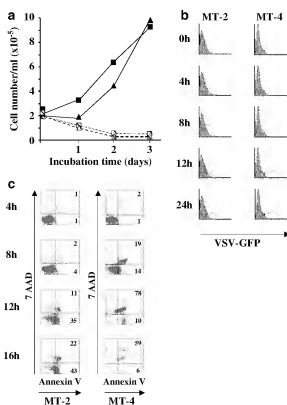


Figure 1 VSV replication in HTLV-1-transformed cells. (a) Growth of VSV-infected MT2 and MT4 cell lines. Cells were mock infected or infected (MOI of 1 PFU/cell) with VSV-HR strain for the indicated times. Cells were stained with acridine orange and ethidium bromide, and viable cells were counted by fluorescence microscopy. (b) Kinetics of VSV-GFP replication in MT-2 and MT-4-transformed cell lines. At the indicated times postinfection, cultures were evaluated for VSV replication by flow cytometry. The full histogram represents GFP expression in mock-infected cells. The empty histogram represents GFP expression in VSV-infected cells. (c) Kinetics of VSV-induced cell death in MT-2 and MT-4 cell lines. At the indicated times, apoptosis was measured using annexin V/7-AAD staining by flow cytometry.

VSV induces caspase-dependent cell death in HTLV-1-transformed cells

VSV-induced cytolytic activity was further confirmed by increased binding of annexin V-FITC to externalized phosphatidylserine of infected but not of control cells. Double staining for annexin V and 7-AAD revealed that phosphatidylserine exposure was induced in a time-dependent manner in both MT-2 and MT-4 cell lines following VSV infection (Figure 1c), and by FACS, the percentage of apoptotic cells at 12 h postinfection increased to 88 and 46% for MT-4 and MT-2, respectively. The number of apoptotic cells in the infected population is higher than the number of VSV-positive cells, suggesting that apoptosis may occur through both direct virus-mediated cytotoxicity and indirect mechanisms involving the release of proapoptotic cytokines.

To address the mechanism of VSV killing in HTLV-1-transformed cells, caspase activation and substrate

cleavage was assessed in MT-4 cells (Woo *et al.*, 1998; Wolf and Green, 1999). As shown in Figure 2a, immunoblot analysis using anti-VSV antiserum revealed significant VSV replication, with VSV G and N protein expression observed as early as 2 h postinfection (Figure 2a, lane 4). Concomitant with viral replication, VSV infection for 4 h was sufficient to cleave procaspase-3 to its enzymatically active p20 and p17 subunits (Figure 2a, lane 5). The enzymatic activity of caspase-3 in VSV-infected cells was further monitored by immunoblot analysis of two caspase-3 substrates, the Rho-GDI, and the DFF45 (Na *et al.*, 1996; Wolf *et al.*, 1999). Both substrates were processed in a time-dependent manner beginning at 4 h postinfection (Figure 2a, lanes 5–9). Furthermore, the use of two broad-spectrum

caspase-3 inhibitors – zVAD and DEVD – significantly delayed the onset of VSV-induced caspase-3 activation and Rho-GDI processing in MT-4 cells (Figure 2b); the 19 and 17 kDa forms of cleaved caspase-3 were weakly detectable at 10 h postinfection in the presence of DEVD (Figure 2b, lanes 8 and 9), while in the presence of zVAD, cleaved caspase-3 was barely detected at 6–12 h (Figure 2b, lanes 13–16). In the presence of both inhibitors, the majority of the 32 kDa procaspase-3 remained uncleaved (Figure 2b). Rho-GDI cleavage in the presence of both inhibitors was also significantly delayed (Figure 2b, lanes 5–8 and 13–16). There was in contrast only a slight delay in the kinetics of VSV replication in the presence of DEVD or zVAD, with VSV G and N protein expression observed at 4 h postinfection (Figure 2b, lanes 4, 11, and 12). Collectively, these data demonstrate that HTLV-1-transformed T cells are permissive for VSV replication, which in turn induces rapid caspase-dependent oncolysis of infected cells.

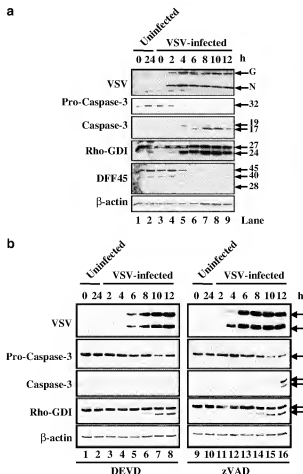


Figure 2 Caspase-dependent cell death in VSV-infected HTLV-1-transformed cells. (a) At the indicated times post-VSV infection (MOI of 1 PFU/cell), the kinetics of caspase-3 activation, Rho-GDI and DFF45 substrate cleavage, and VSV G and N protein expression were assessed in 20 µg of whole-cell lysates using SDS-PAGE and immunoblot analysis with β-actin as a loading control. Similar results were gathered from three independent experiments. (b) Caspase inhibitors zVAD and DEVD (100 µM final) were added to MT-4 cells 20 min prior to VSV infection and at different times postinfection cell lysates were analysed for caspase-3 activation, Rho-GDI and DFF45 substrate cleavage, and VSV G and N protein expression, as above.

VSV replicates and induces rapid cell death in primary ATL cells

To investigate the *ex vivo* responses of primary ATL cells to VSV infection, PBMCs were obtained from four patients with acute ATL disease; samples were collected at the time of diagnosis before chemotherapy. The survival time of ATL-1, -2, -3, and -4 patients were 40 days, 30 days, > 9 months, and 14 months, respectively. Hematological analysis revealed that PBMCs from ATL-1, -2, -3, and -4 patients contained substantial leukemic cells: 79, 39, 77, and 40% atypical lymphocytes, respectively (Table 1). Following Ficoll purification and culture, the percentage of CD4+CD8-CD25+ T leukemic cells ranged between 76 and 89%. In parallel, PBMCs were collected from two patients with HAM/TSP, and a healthy volunteer (naïve PBMC) (Table 1). Cells (ATL, HAM/TSP, and normal PBMC) were mock infected or infected with the rVSV-GFP (MOI of 1.0 PFU/cell) for various times and the level of GFP expression (as a measure of virus replication) was examined by flow cytometry. GFP-expressing cells (10–15%) were detected in all ATL samples beginning at 24 h and the percentage of GFP-positive cells increased in a time-dependent manner (30–40% at 72 h) (Figure 3a, and data not shown). On the other hand, no increase in GFP expression was detected in HAM/TSP or naïve PBMC even after 72 h of VSV infection (Figure 3a), illustrating the permissiveness of VSV in ATL cells. VSV induced dramatic cell death (> 50% at 24 h and > 70% at 72 h) only in ATL cells but not in HAM/TSP or naïve PBMC (Figure 3b). In parallel, immunoblot analysis from ATL-infected cells clearly revealed time-dependent processing of procaspase-3 and Rho-GDI at 24 h postinfection, concomitant with viral replication (Figure 3c). High levels of apoptotic ATL cells at 24 and 72 h again indicates that cell death may be occurring through both direct virus-mediated and indirect apoptotic mechanisms.

Table 1 Clinical and biological features of ATL patients and PBMC death following *ex vivo* infection with VSV

Patient	Sex	Age (years)	Type of leukemia	Survival	Chemotherapy at sampling	Circulating leukemic cells (%)	Increase in % annexin V-positive cells at 48 h in VSV- versus mock-infected PBMCs
ATL-1	M	39	ATL (acute form)	40 days	Untreated	79	55
ATL-2	F	51	ATL (acute form)	30 days	Untreated	39	50
ATL-3	F	60	ATL (acute form)	> 9 months	Untreated	77	56
ATL-4	M	41	ATL (acute form)	14 months	Untreated	40	62
CLL-1	M	60	B-CLL	> 10 years	Chlorambucil	90	0
CLL-2	M	83	B-CLL	> 10 years	Chlorambucil	45	6
CLL-3	F	87	B-CLL	> 5 years	Untreated	72	3
CLL-4	M	85	T-CLL	> 5 years	Untreated	75	7
HAM/TSP-1	M	48	—	—	—	—	13
HAM/TSP-2	F	69	—	—	—	—	3
Healthy control	M	42	—	—	—	—	6

At 48 h postinfection, mock- and VSV-infected cells were analysed for annexin V staining by flow cytometry. The VSV-induced cell death was estimated by the difference between the percentages of annexin V-positive cells in the VSV- and mock-infected samples. Data from CLL, HAM/TSP and healthy controls are indicated.

VSV replicates and induces cell death in CLL cell lines but not in primary CLL lymphocytes

To investigate the specificity of VSV oncolysis, the ability of VSV to replicate and lyse two other leukemic lines, I-83 (Epstein-Barr virus-transformed B-CLL) and WSU (Epstein-Barr virus-negative B-CLL) cell lines, was evaluated. Similar to HTLV-1-infected cell lines, almost no viable I-83 or WSU cells were detected 48 h post-VSV infection (Figure 4a). As shown in Figure 4b, both I-83 and WSU were GFP positive (> 70 and 40%, respectively) at 24 h after rVSV-GFP infection. The time-dependent induction of cell death was confirmed by annexin V and 7-AAD staining in infected but not control cells, and revealed extensive cell death (60 and 76%, respectively) as early as 16 h postinfection (Figure 4c). VSV replication was then assessed in primary cells from three B-CLL and one T-CLL patients, with the samples containing more than 60% leukemic cells. In contrast to I-83 and WSU cell lines, primary CLL cells infected *ex vivo* under similar conditions were not permissive to VSV replication (Figure 4d). Furthermore, VSV infection of primary CLL cells showed no increase in cell death as measured by annexin V staining (Table 1), although these CLL samples were efficiently killed by chlorambucil, etoposide or camptothecin (data not shown). Similar results were observed for primary CLL cells at an MOI as high as 100 PFU/cell. These data emphasize the *ex vivo* specificity of VSV oncolytic activity in primary ATL but not primary CLL cells.

Activation of CD4 T-lymphocytes renders them susceptible to VSV replication and cell death

Previous studies have demonstrated that activation of the IL-2/IL-2R loop during the acute phase of HTLV-1 infection contributes to the activation and proliferation of HTLV-1-infected CD4+ CD25+ subset of ATL cells (Maruyama et al., 1987; Satoh et al., 2002). Thus, we next examined the effect of cell activation on VSV

permissiveness and cytotoxic activity. CD4+ T-lymphocytes were mock activated or activated with anti-CD3/CD28 (5 µg/ml) and IL-2 (70 U/ml) for 24 h, followed by infection with VSV (MOI of 1.0 PFU/cell) for different time intervals. The activation status of CD4+ T cells was first analysed by flow cytometry against CD25 and CD69 activation markers at the time of VSV infection (Figure 5a). VSV infection of anti-CD3/CD28-activated primary CD4+ T cells resulted in VSV replication (Figure 5c, right panel) compared to nonstimulated cells (Figure 5c, left panel), indicating a relationship between T-cell activation status and VSV replication. Furthermore, VSV replication in anti-CD3/CD28-activated, CD4+ lymphocytes resulted in significant cell death by 48 h postinfection with more than 50% of the cells in the sub-G fraction, whereas in unstimulated primary CD4+ T cells, the sub-G fraction was only 7%. Treatment with anti-CD3/CD28 or VSV alone modestly increased the sub-G proportion to 15% (Figure 5b). In another experiment, PMA treatment of CD4+ T cells induced significantly higher levels of cell activation compared to anti-CD3/CD28 stimulation (CD69 positivity ~ 99 versus ~ 33%, respectively) and was accompanied by extensive VSV replication and cell death (data not shown).

ATL cells are more permissive to VSV replication than activated CD4 T-lymphocytes

Next, the replicative capacity of VSV was examined in ATL cells versus CD4 T-lymphocytes activated to similar levels. For this purpose, anti-CD3/CD28 was used to optimize the intensity of T-cell activation, using an ATL sample that was 86% CD4+, and a primary T-lymphocyte sample that was 96% CD4+. Activation of CD4+ T cells with anti-CD3/CD28 increased the CD25 expression level (~ 32% with 2.5 µg/ml of anti-CD3 and ~ 38% with 5 µg/ml of anti-CD3) comparable to the CD25 level detected for ATL cells, 46% (Figure 6a). Following VSV infection for 24 and 48 h, immunoblot

analysis demonstrated that VSV replication in activated CD4⁺ T-lymphocytes was time dependent and directly related to the intensity of cell activation (Figure 6b). Nevertheless, ATL cells showed fourfold higher levels of viral G and N proteins at 24h after infection compared to activated CD4⁺ T cells under similar conditions, indicating that ATL leukemic cells were more permissive to VSV than activated, primary T-lymphocytes.

To further evaluate T-cell activation and VSV permissiveness, anti-CD3 and anti-CD28 were used

separately to stimulate the CD4⁺ T-cell population (Figure 6c). Clearly, CD3 stimulation of the CD3 pathway was sufficient to render T cells permissive to VSV replication (Figure 6c, lanes 3 and 4), whereas stimulation of the CD28 pathway had little effect on the capacity of the T-cell population to support VSV replication (Figure 6c, lanes 5-7). As expected, the combination of CD3/CD28 rendered CD4⁺ T cells permissiveness for VSV replication, but with minimal additive effect compared to CD3 stimulation alone (Figure 6c, compare lanes 3 and 4 with lanes 9 and 10). In related experiments, no change in the expression of translational components eIF2 α , B or Be (data not shown) was observed, as described previously (Balachandran and Barber, 2004). Thus, a critical component of the ability of VSV to replicate in CD4⁺ T-lymphocytes is the stimulation of the TCR/CD3 activation pathway.

Discussion

Oncolytic viruses provide an attractive potential cancer therapeutic because of their ability to replicate selectively within tumor but not normal cells. Several oncolytic viruses have demonstrated promising results in preclinical and early clinical studies (Kirm et al., 2001; Chiocca, 2002; Hawkins et al., 2002; Lichty et al., 2004), including naturally attenuated replication competent viruses such as reovirus (nonenveloped, double-stranded (ds)RNA), parvovirus (nonenveloped, single-stranded DNA), Newcastle disease virus, measles virus, and VSV (Gromeier and Wimmer, 2001; Kirm et al., 2001; Norman et al., 2001; Bell et al., 2002; Hawkins et al., 2002). The present study provides the first conclusive evidence that VSV can replicate selectively in and cause extensive caspase-dependent oncolysis of primary *ex vivo* ATL cells. Importantly, VSV infection did not induce cell death in naive primary CD4⁺ T-lymphocytes from healthy volunteers or in PBMCs from patients with HAM/TSP.

VSV specifically replicates in tumor cells due to the attenuation of the antiviral IFN response, a phenotype

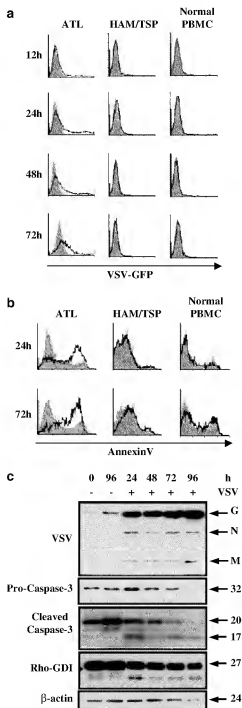


Figure 3 VSV oncolysis of primary ATL cells. (a) Kinetics of VSV replication in ATL cells. ATL, HAM/TSP, and normal PBMC were isolated as described in Materials and Methods. Cells were mock infected or infected (MOI of 1 PFU/cell) with rVSV-GFP recombinant virus. At the indicated times, cells were analysed for VSV replication by flow cytometry. The full histogram represents GFP expression in mock-infected cells. The empty histogram represents GFP expression in VSV-infected cells. (b) Induction of cell death in ATL cells. At 24 and 72 h postinfection, mock- and VSV-GFP-infected cells were analysed for annexin V staining by flow cytometry. The full histogram represents mock-infected cells. The empty histogram represents VSV-infected cells. (c) Kinetics of caspase-3 activation and VSV replication in ATL cells. At the indicated times postinfection, caspase-3 activation, Rho-GDI processing, and VSV replication were detected in 20 μ g of whole-cell lysates by SDS-PAGE and immunoblot analysis, with β -actin as a loading control. Similar results were gathered from four patient samples.

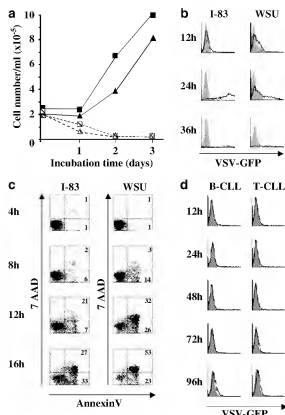


Figure 4 VSV replication in CLL cells. (a) Kinetics of VSV-induced cell death in I-83 and WSU CLL cell lines. Cells were mock infected or infected (MOI of 1 PFU/cell) with VSV-HR strain for the indicated times. Cells were stained with acridine orange and ethidium bromide, and viable cells were counted by fluorescence microscopy. (■) I-83; (□) I-83 + VSV; (▲) WSU; and (△) WSU + VSV. (b) VSV replication in I-83 and WSU B-CLL cell lines. (b) At the indicated times postinfection, cultures were evaluated for VSV replication by flow cytometry. The full histogram represents GFP expression in mock-infected cells. The empty histogram represents GFP expression in VSV-infected cells. (c) Kinetics of VSV-induced cell death in I-83 and WSU cell lines. At the indicated times, apoptosis was measured using annexin V/7-AAD staining by flow cytometry. (d) VSV infection in primary CLL lymphocytes. B-CLL and T-CLL PBMC were isolated as described in Materials and methods. At the indicated times postinfection, cultures were evaluated for VSV replication by flow cytometry. The full histogram represents GFP expression in mock-infected cells and the empty histogram represents GFP expression in VSV-infected cells.

that frequently arises during tumor evolution (Linge *et al.*, 1995; Sun *et al.*, 1998; Matin *et al.*, 2001). The induction of type I IFN- α/β is a potent host defense mechanism against viral infection, and many viruses have evolved strategies to overcome the antiviral effects of this cytokine. However, VSV is extremely sensitive to the antiviral actions of IFN (Belkowski and Sen, 1987); hence, VSV infection in humans is asymptomatic in most cases or results in a mild febrile illness. In contrast, mice harboring a defective IFN system are highly sensitive to normally harmless exposure of VSV (Muller *et al.*, 1994; Durbin *et al.*, 1996). Stojdl *et al.* (2003)

demonstrated recently that 80% of the NCI 60 cell line collection (Weinstein *et al.*, 1997) is permissive for VSV infection. Indeed, most of these cells were found to have impaired responses to either IFN- α or IFN- β (Stojdl *et al.*, 2003). In the context of the present study, we observed that IFN production in ATL cells is defective compared to primary T cells (S Olieri *et al.*, unpublished) and experiments are underway to examine the relationship between IFN induction and VSV replication.

Subsequent biochemical analysis provided evidence that translation control downstream of PKR activation, frequently dysregulated in many transformed cells, can cooperate with the attenuated IFN antiviral activity to facilitate VSV oncolysis (Balachandran and Barber, 2004). Elevated levels of eIF2 β are required for increased permissiveness of transformed cells to VSV replication. Cells transfected with siRNA against eIF2 β were almost completely protected against VSV-induced cytolysis and produced 10-fold lower yield of virus than control cells (Balachandran and Barber, 2004). In the present study, no evidence of changes in the expression of eIF2 β was obtained in resting or activated CD4+ T-lymphocytes, suggesting that alternative mechanisms may be operative in the T-cell context. In this regard, triggering the TCR/CD3 activation pathway was sufficient to render normal CD4+ T-lymphocytes permissive for VSV replication. This preliminary observation suggests that dissection of the CD3 pathway may identify critical downstream regulators of VSV replication and/or susceptibility to oncolysis. Since activation of Ras/Raf signaling is downstream of CD3 activation, the possibility exists that activated Ras may be required for VSV oncolysis, as previously demonstrated for Reovirus-induced oncolysis (reviewed in Norman *et al.*, 2001).

Interestingly, nonleukemic cells from patients with HAM/TSP were resistant to VSV infection. Since HAM/TSP cells are known to spontaneously proliferate *in vitro* (Cavrois *et al.*, 1996; Mortreux *et al.*, 2003), an important caveat to consider in the interpretation of the present results is that the HTLV-1-infected subpopulation (1–5%) within the HAM/TSP PBMC samples may in fact be susceptible to VSV oncolysis. It is well documented that HTLV-1 infection, and specifically the viral Tax oncoprotein, leads to T-cell activation via an IL-2/IL-2 receptor autocrine loop, as well as induction of the NF- κ B and CREB pathways (Yoshida, 2001; Franchini *et al.*, 2003); activation of these Tax-mediated pathways may also influence the susceptibility of the HTLV-1-infected subpopulation in HAM/TSP to VSV infection. It will be of interest to determine the differential sensitivities of the HTLV-1-infected and -uninfected compartments in HAM/TSP.

Programmed cell death represents the convergence of multiple apoptotic pathways from numerous distinct initiating events and insults (Eastman and Rigas, 1999; Nguyen and Wells, 2003). While many of the details of direct and indirect mechanisms of VSV-induced apoptosis remain to be determined, the role of viral replication in VSV oncolysis is also controversial. It

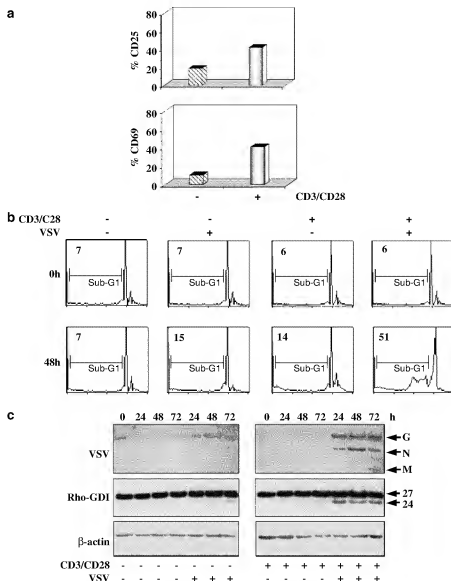


Figure 5 VSV-induced oncolysis in activated CD4 T-lymphocytes. Isolated CD4⁺ T-lymphocytes were incubated with anti-CD3/anti-CD28/IL-2 or PMA for 24 h. (a) Expression of CD25 and CD69 activation markers were assessed by flow cytometry at the time of VSV infection to determine the CD4⁺ T-lymphocyte activation status. (b) Activated and nonactivated CD4⁺ lymphocytes were mock infected or infected (MOI of 1 PFU/cell) with VSV-IR strain for the indicated times postinfection. Apoptosis was determined by quantification of the sub-G₀ population (1×10^4 cells/measurement). (c) Kinetics of VSV replication and Rho-GDI processing in activated and nonactivated CD4⁺ T-lymphocytes. VSV replication and Rho-GDI processing were detected in 20 μ g of whole-cell lysates by SDS-PAGE and immunoblot analysis, with β -actin as a loading control.

has been reported that VSV-induced cell death occurs at early stages after infection, and requires viral particle internalization and uncoating, but not viral replication or *de novo* viral protein synthesis (Gadaleta *et al.*, 2002). In contrast, other studies demonstrated that VSV-induced apoptosis correlates with viral protein expression, and that activation of caspase-3-like proteases was required for VSV-induced apoptosis but not viral replication (Hobbs *et al.*, 2001, 2003). In addition, VSV matrix M protein has been shown to inhibit host cell gene expression and nuclear export of host mRNA via an association between M protein and Nup98, a host

nuclear pore protein, with subsequent induction of apoptosis (Kopecky *et al.*, 2001; Desforges *et al.*, 2002; Kopecky and Lyles, 2003). The present results in HTLV-1-transformed MT-4 cells and in primary ATL demonstrate that VSV-induced apoptosis correlated with VSV replication, and that caspase-3 activation and subsequent apoptotic events involving Rho-GDI cleavage and DFF45 processing occurred only following VSV replication. Nonetheless, both direct virus-mediated apoptotic mechanisms and indirect mechanisms involving proapoptotic cytokine release may be involved in ATL killing by VSV.

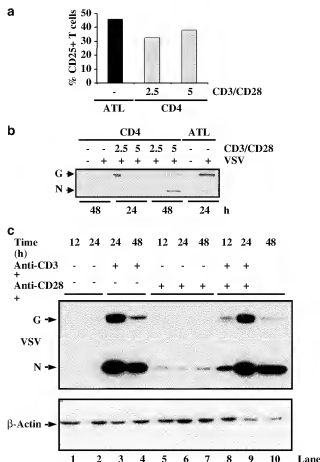


Figure 6 Enhanced VSV replication in ATL versus activated CD4 T-lymphocytes. (a) Activation of CD4 T-lymphocytes. Purified isolated CD4 T-lymphocytes were incubated with 2.5 or 5.0 µg/ml of anti-CD3 plus anti-CD28/IL-2 for 24 h and CD25 expression levels were assessed by flow cytometry at 24 h after treatment. (b) Kinetics of VSV replication in CD4 and ATL lymphocytes. Activated CD4 lymphocytes and ATL cells were mock infected or infected (VSV-HR, MOI of 1 PFU/cell), and at the indicated times postinfection, total cell lysates were prepared and VSV G and N protein expression was analysed in 20 µg of total cell lysates by SDS-PAGE and immunoblot analysis. (c) CD3 activation of CD4+ T-lymphocytes. Purified isolated CD4+ T-lymphocytes were incubated with 5.0 µg/ml of anti-CD3 monoclonal antibody or anti-CD28 monoclonal antibody for 24 h and then infected with VSV at an MOI of 1 PFU/cell. At the indicated times postinfection, total cell lysates were prepared and VSV G and N protein expression was analysed in 20 µg of total cell lysates by SDS-PAGE and immunoblot analysis with β-actin as a loading control.

Based on the discrepancies between VSV killing of established hematopoietic cells and primary leukemic cells documented in the present study, the oncolytic

activity of VSV in immortalized cell lines may not systematically reflect VSV killing in their primary cancer counterparts, thus underscoring the need to assess the sensitivity of primary cancer cells to virotherapy. While CLL cell lines were permissive for viral replication, primary CLL cells were resistant to VSV replication and subsequent cell death. This discrepancy in VSV oncolysis may be due to the fact that CLL cells do not proliferate but remain in G_0 *ex vivo* (Meinhardt *et al.*, 1999; Caligaris-Cappio, 2003). That VSV oncolysis may be linked to the state of cell growth or activation was addressed in primary T cells by demonstrating that TCR/CD3 activation enhanced permissiveness to VSV replication. It should be noted, however, that even in activated T cells, viral replication was about fourfold higher in ATL cells than in activated CD4 cells, further supporting the idea that VSV replication may be influenced by genetic defects unique to tumor cells, including deficiencies in antiviral IFN- α/β and p53 signaling pathways (Stojdl *et al.*, 2000, 2003; Takaoka *et al.*, 2003). Thus, p53 dysfunction, myc overexpression, or p16^{INK4a} inactivation in ATL cells (Yoshida, 2001; Matsuoka, 2003) could increase the susceptibility of ATL cells to VSV and other oncolytic viruses (Balachandran *et al.*, 2001; Chiocca, 2002).

Although we provide information on the *ex vivo* oncolytic potential of VSV in primary ATL cells, we cannot predict the oncolytic efficiency of VSV *in vivo* before the onset of potentially neutralizing antibody response. Interestingly, naturally occurring VSV variants with a mutated matrix protein unable to block the IFN production in infected cells have been shown to retain oncolytic activity in a variety of *in vivo* models (Stojdl *et al.*, 2003). The possibility to enhance the oncolytic activity of VSV using genetic engineering to insert immunomodulatory or suicide cassettes has also been demonstrated both *in vitro* and *in vivo* models (Fernandez *et al.*, 2002). These findings further highlight the potential of VSV in cancer virotherapy and emphasize the importance of future investigations in primary *ex vivo* tumor cells.

Acknowledgements

We thank the members of the Molecular Oncology Group, Lady Davis Institute, and McGill University for helpful discussions. We also thank Lama Fawaz for helpful discussions. This research was supported by grants from the National Cancer Institute with funds from the Canadian Cancer Society to JH and JB, the Canadian Institutes of Health Research and CANVAC, and the Canadian Network for Vaccines and Immunotherapeutics (to JH). ESA is supported by a Fellowship from the FRSQ and JH is a recipient of a CIHR Senior Investigator award.

References

- Balachandran S, Barber GN. (2000). *IUBMB Life* 50: 135–138.
- Balachandran S, Barber GN. (2004). *Cancer Cell* 5: 51–65.
- Balachandran S, Porosnicu M, Barber GN. (2001). *J Virol* 75: 3474–3479.

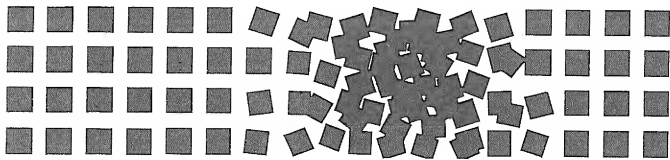
- Bazarbachi A, Ghez D, Lepelletier Y, Nasr R, De The H, El Sabban ME *et al.* (2004). *Lancet Oncol* 5: 664–672.
- Belkowsky LS, Sen GC. (1987). *J Virol* 61: 653–660.
- Bell JC, Garson KA, Lichty BD, Stojdl DF. (2002). *Curr Gene Ther* 2: 243–254.

- Caligaris-Cappio F. (2003). *Br J Haematol* **123**: 380–388.
- Cavrois M, Gessain A, Wain-Hobson S, Wattel E. (1996). *Oncogene* **12**: 2419–2423.
- Chioce EA. (2002). *Nat Rev Cancer* **2**: 938–950.
- Desforges M, Despars G, Berard S, Gosselin M, McKenzie MO, Lyles DS et al. (2002). *Virology* **295**: 63–73.
- Durbin JE, Hackenmiller R, Simon MC, Levy DE. (1996). *Cell* **84**: 443–450.
- Eastman A, Rigas JR. (1999). *Semin Oncol* **26**: 7–16.
- Ebert O, Shinozaki K, Huang TG, Savantaus MJ, Garcia-Sastre A, Woo SL. (2003). *Cancer Res* **63**: 3605–3611.
- Fernandez M, Porosnicu M, Markovic D, Barber GN. (2002). *J Virol* **76**: 895–904.
- Franchini G, Nicot C, Johnson JM. (2003). *Ade Cancer Res* **89**: 69–132.
- Gadaleta P, Vacotto M, Coulombie F. (2002). *Virus Res* **86**: 87–92.
- Gessain A, Barin F, Vernant JC, Gout O, Maurs L, Calender A et al. (1985). *Lancet* **2**: 407–410.
- Grigera PR, Garcia-Briones M, Periolo O, la Torre JL, Wagner RR. (1996). *J Virol* **70**: 8492–8501.
- Gromeier M, Wimmer E. (2001). *Curr Opin Mol Ther* **3**: 503–508.
- Hawkins LK, Lemoine NR, Kirn D. (2002). *Lancet Oncol* **3**: 17–26.
- Hinuma Y, Komoda H, Chosa T, Kondo T, Kohakura M, Takenaka T et al. (1982). *Int J Cancer* **29**: 631–635.
- Hobbs JA, Hommel-Berrey G, Brahmi Z. (2003). *Hum Immunol* **64**: 82–92.
- Hobbs JA, Schloemer RH, Hommel-Berrey G, Brahmi Z. (2001). *Virus Res* **80**: 53–65.
- Huang TG, Ebert O, Shinozaki K, Garcia-Sastre A, Woo SL. (2003). *Mol Ther* **8**: 434–440.
- Kirn D, Martuza RL, Zwiebel J. (2001). *Nat Med* **7**: 781–787.
- Kopecky SA, Lyles DS. (2003). *J Virol* **77**: 5524–5528.
- Kopecky SA, Willingham MC, Lyles DS. (2001). *J Virol* **75**: 12169–12181.
- Lichty BD, Stojdl DF, Taylor RA, Miller L, Frenkel I, Atkins H et al. (2004). *Hum Gene Ther* **15**: 821–831.
- Linge C, Gewert D, Rossmann C, Bishop JA, Crowe JS. (1995). *Cancer Res* **55**: 4099–4104.
- Mariyama M, Shibuya H, Harada H, Hatakeyama M, Seiki M, Fujita T et al. (1987). *Cell* **48**: 343–350.
- Matin SF, Rackley RR, Sadhukhan PC, Kim MS, Novick AC, Bandyopadhyay SK. (2001). *Cancer Res* **61**: 2261–2266.
- Matsuoka M. (2003). *Oncogene* **22**: 5131–5140.
- Meinhardt G, Wendtner CM, Hallek M. (1999). *J Mol Med* **77**: 282–293.
- Mortreux F, Gabet AS, Wattel E. (2003). *Leukemia* **17**: 26–38.
- Muller U, Steinhoff U, Reis LF, Hemmi S, Pavlovic J, Zinkernagel RM et al. (1994). *Science* **264**: 1918–1921.
- Na S, Chuang TH, Cunningham A, Turi TG, Hanke JH, Bokoch GM et al. (1996). *J Biol Chem* **271**: 11209–11213.
- Nguyen JT, Wells JA. (2003). *Proc Natl Acad Sci USA* **100**: 7533–7538.
- Norman KL, Farassati F, Lee PW. (2001). *Cytokine Growth Factor Rev* **12**: 271–282.
- Obuchi M, Fernandez M, Barber GN. (2003). *J Virol* **77**: 8843–8856.
- Osame M, Usuku K, Izumo S, Ijichi N, Amitani H, Igata A et al. (1986). *Lancet* **1**: 1031–1032.
- Satoh M, Toma H, Sugahara K, Etoh K, Shiroma Y, Kiyuna S et al. (2002). *Oncogene* **21**: 2466–2475.
- Sharif-Askari E, Alam A, Rheume E, Beresford PJ, Scotto C, Sharma K et al. (2001). *EMBO J* **20**: 3101–3113.
- Shimoyama M. (1991). *Br J Haematol* **79**: 428–437.
- Steele TA. (2000). *Proc Soc Exp Biol Med* **223**: 118–127.
- Stojdl DF, Lichty B, Knowles S, Marius R, Atkins H, Sonenberg N et al. (2000). *Nat Med* **6**: 821–825.
- Stojdl DF, Lichty BD, tenOver BR, Paterson JM, Power AT, Knowles S et al. (2003). *Cancer Cell* **4**: 263–275.
- Sun WH, Pabon C, Alsayed Y, Huang PP, Jandeska S, Uddin S et al. (1998). *Blood* **91**: 570–576.
- Takaoka A, Hayakawa S, Yanai H, Stoiber D, Negishi H, Kikuchi H et al. (2003). *Nature* **424**: 516–523.
- Uchiyama T, Yodoi J, Sagawa K, Takatsuki K, Uchino H. (1977). *Blood* **50**: 481–492.
- Weinstein JN, Myers TG, O'Connor PM, Friend SH, Fornace Jr AJ, Kohn KW et al. (1997). *Science* **275**: 343–349.
- Wolf BB, Green DR. (1999). *J Biol Chem* **274**: 20049–20052.
- Wolf BB, Schuler M, Echeverri F, Green DR. (1999). *J Biol Chem* **274**: 30651–30656.
- Woo M, Hakem R, Soengas MS, Duncan GS, Shahinian A, Kagi D et al. (1998). *Genes Dev* **12**: 806–819.
- Wu JT, Byrne HM, Kirn DH, Wein LM. (2001). *Bull Math Biol* **63**: 731–768.
- Yasunaga J, Matsuoka M. (2003). *Int J Hematol* **78**: 312–320.
- Yoshida M. (2001). *Annu Rev Immunol* **19**: 475–496.

CANCER

*Principles & Practice
of Oncology*

6th Edition



www.LWWoncology.com

2001



LIPPINCOTT WILLIAMS & WILKINS

A Wolters Kluwer Company

Philadelphia • Baltimore • New York • London
Buenos Aires • Hong Kong • Sydney • Tokyo



EDITED BY

Vincent T. DeVita, Jr., MD

Director, Yale Cancer Center; Professor of Medicine, Professor of Epidemiology and Public Health, Yale University School of Medicine, New Haven, Connecticut

Samuel Hellman, MD

A. N. Pritzker Distinguished Service Professor, Department of Radiation and Cellular Oncology, University of Chicago, Chicago, Illinois

Steven A. Rosenberg, MD, PhD

Chief of Surgery, National Cancer Institute, National Institutes of Health; Professor of Surgery, Uniformed Services University of the Health Sciences School of Medicine, Bethesda, Maryland; Professor of Surgery, George Washington University School of Medicine, Washington, DC

319 Contributing Authors

SECTION 2

Cancer Drug Development

EDWARD CHU
MICHAEL R. GREYER
BRUCE A. CHABNER

Identification and Screening of New Agents

More than five decades of research effort in cancer drug discovery and development have provided approximately six dozen approved products for the treatment of malignancy.^{1,2} Although major advances have been made in the chemotherapeutic management of some patients, particularly in hematologic malignancies, one-half of all cancer patients either do not respond to therapy or relapse from the initial response and ultimately die from their metastatic disease. Thus, the continued commitment to the arduous task of discovering new cancer therapeutic agents remains critically important.³ Many of the existing antineoplastic agents share a common mechanism of action. Current research efforts are more diverse than ever, being driven by explosive discoveries in molecular biology and related areas to fully elucidate the development of the malignant process (e.g., factors controlling tumor angiogenesis and metastatic potential). The hope for improvement in treatment outcome for most patients with metastatic disease resides in continued research designed to discover novel therapeutic products that exploit differences in molecular targets between normal and tumor cells and to use them in combination with biologic agents and immune therapies to eradicate systemic disease not curable by surgery or irradiation.

Beyond the intellectual challenge of drug discovery, formidable effort, time, and expense are required for the complex development processes that move a new agent from discovery to its ultimate approval for use in the treatment of malignancy. Numerous pitfalls may threaten the progress of a promising agent (e.g., excessive early toxicity, ineffective route or schedule of administration, inappropriate formulation, long-term unpredicted toxicities, and delays in the execution of clinical trials). Although the time to drug approval for the treatment of cancer has varied considerably, depending on the specific agent (e.g., 6 to 12 years from the time of initiation of clinical trials), efforts are being made to expedite both the preclinical and clinical components of investigation. In other areas of medicine, the time to develop specific drugs may be equally long and difficult. However, the potentially fatal consequences of unsuccessful treatment of this disease continue to impart urgency in the discovery and development of novel anticancer agents.

DRUG DISCOVERY

HOW DRUGS ARE DISCOVERED

This section considers strategies for identifying new chemical entities, whether they be synthesized chemicals or compounds extracted or derived from plant, microbial, and

marine animal sources. The parallel process for discovery and development of biologic agents is discussed elsewhere in this text. (See Chapter 18.)

In establishing a program for drug discovery, cancer researchers must address two fundamental questions: What screening system should be used to detect a compound of interest? What compounds should be tested in this system? The answers to these questions determine whether the research effort is empiric, with few preconceived notions about where to search for compounds and what to use as the screen, or whether it focuses on a specific biologic target, such as an oncogene, and tests a specific set of materials, such as natural products and rationally synthesized inhibitors of a target enzyme. The history of cancer drug discovery reflects an evolution from highly empiric approaches, based on testing of randomly selected compounds against rapidly proliferating murine leukemia, to the current, more focused testing of natural products, rationally synthesized agents, and biologic products against well-characterized tumor cell lines or molecular targets. Even in its earliest days, however, cancer drug discovery attracted scientists who had a theoretical basis for testing certain types of compounds. Perhaps the two best examples are the antifolates, initially tested by Farber et al.,⁴ and the fluoropyrimidines synthesized by Heidelberger and colleagues⁵ (see Chapter 19.5).

The story of the discovery of antifolates is particularly instructive because it illustrates the important interplay between cancer biology and drug discovery. The earliest uses of an antifolate as a chemotherapeutic agent resulted from the astute observations of Farber and associates,⁴ who observed an acceleration of the leukemic process in patients being treated with folic acid. A series of folic acid antagonists were provided to Farber and colleagues by the medicinal chemists at Lederle Laboratories. Although structure-activity relations of antifolates and the intracellular target of these compounds were unknown at that time, it was clear from laboratory studies that modified folates could inhibit tumor cell growth. The initial clinical trial involved the administration of pteroylaspartic acid (an analogue of folic acid, or pteroylglutamate) to a nonruminant patient with progressive acute myelogenous leukemia, which resulted in a markedly hypocellular bone marrow without actually producing clinical benefit. The investigators were sufficiently encouraged, however, to administer a more powerful folic acid antagonist, aminopterin (2,4-diaminopteroylglutamate), to children with advanced stages of acute leukemia. Substitution of an amino group at the 4 position of the folate pteridine ring created a tight-binding inhibitor of dihydrofolate reductase and yielded drugs with the potential to induce remissions. Approximately 10 of the first 16 patients treated with aminopterin demonstrated evidence of hematologic and clinical improvement. These early clinical experiences provided the foundation for medicinal chemists to synthesize a number of agents with structural similarities to naturally occurring folates. Moreover, these studies revealed that various substitutions resulted in different sites of action, in addition to inhibition of dihydrofolate reductase (Fig. 19.2-1).

From these relatively primitive beginnings, rational design efforts have progressed to the use of computer modeling of drug-enzyme interactions as the basis for cancer drug discovery. Advances in x-ray crystallographic and nuclear magnetic resonance structural characterization of ligands and their target molecules have significantly enhanced the potential for

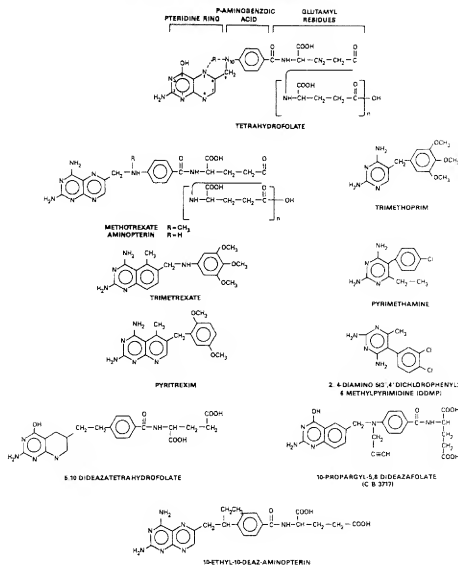


FIGURE 19.2-1. Structure of tetrahydrofolate and clinically useful antifolate compounds. (From Chabner BA, Collins JM, eds. *Cancer chemotherapy and biophysics: principles and practice*. Philadelphia: Lippincott-Raven, 1996:112, with permission.)

rational design and, as is described later (see the section Molecular-Targeted Screening), such screening efforts are now beginning to identify effective small molecules with efficacy against various human malignancies. Symmetric inhibitors of the protease of human immunodeficiency virus type 1 that were designed on the basis of the three-dimensional symmetry of the active enzyme site are currently in clinical use, thus demonstrating the feasibility and merit of such an approach.⁶

In most current drug discovery efforts, the rational and empiric approaches are being combined. Lead compounds are identified as inhibitors for molecular targets through molecular screening.⁷ The lead compound can then be modified or enhanced by chemical analogue synthesis based on a variety of considerations, including a detailed study of target-inhibitor interaction. The complete characterization of the target and its interaction with the lead agent provides the basis for enhancing drug-target interaction. A key decision in this approach is the selection of a suitable target that is likely to have an impact on clinical outcome (enzyme, growth factor receptor, or oncogene product). The next challenge is the development of an appropriate and practical assay to identify the actual lead agents.

Although early efforts in cancer drug discovery tested agents either from the broad universe of synthetic chemicals or from a more targeted rational effort, attention increasingly has focused on natural products as an important, untapped source of promising lead compounds with unique sites of action as antineoplastic agents.⁸ The enormous diversity and complexity of chemical entities that have evolved as part of nature's chemical warfare cannot be readily duplicated by compounds synthesized in the laboratory and be made available for screening. However, the technological advances in combinatorial approaches for synthesizing large numbers of complex substances have provided an entire new source for novel antineoplastic agents.^{9,10} Combinatorial chemistry can provide two different kinds of libraries that can then be used for further drug development.¹¹⁻¹² The first is a generic library, which is used to discover a novel structural motif or feature that possesses a certain biologic activity. The goal of such an unbiased library is to identify a completely novel lead compound. The second type is a focused, biased library that serves to fine-tune the properties and biologic activity of an existing lead compound. In this case, the objective is to identify new lead com-

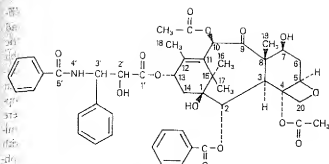


FIGURE 19.2-2. Structure of Taxol. (From Chabner BA, Collins JM, eds. *Cancer chemotherapy and biotherapy: principles and practice*. Philadelphia: Lippincott-Raven, 1996, with permission.)

ounds based on known structures that have already proven to be biologically active.

Approximately 30% of the currently effective antineoplastic agents are from natural sources or are derivatives of a natural product lead.¹⁴ Certain themes run through the efforts to discover and develop natural products. Active compounds often have exceedingly complex structures that complicate efforts at total synthesis. Problems of supply and dependence on a natural resource, therefore, must be anticipated. Structure-activity relations are difficult to elucidate because of the basic problems presented by the unusual chemistry of these compounds and by the multiple chiral centers in these molecules (Fig. 19.2.2). However, the overall contribution of these complex chemical entities to the management of patients has been extremely rewarding.

Among the natural products, microbial antibiotics have been the most important source of cytotoxic agents. As a result of the great advances in the field of microbiology during the 1940s and the dawn of effective antibiotic therapy, potent anticancer drugs were sought in fermentation broths obtained from soil microbes, including bacteria, fungi, and related organisms. The discoveries of the actinomycins, anthracyclines, bleomycin, doxorubicin, and other agents have contributed valuable new entities to the repository of effective antineoplastic agents. Natural product drug discovery, however, must be complemented by efforts to improve leads through chemical modification and analogue synthesis. The discovery and subsequent clinical development of anthracyclines highlights the need for the close interplay of chemistry, biology, and clinical pharmacology in producing improved anticancer agents.

Doxorubicin, isolated from a colony of *Streptomyces* in 1957, eventually was demonstrated to have significant antileukemic activity in patients.¹⁵ Additional research to induce mutant strains of the fungus *Streptomyces* resulted in the isolation of doxorubicin. Although the difference between these two anthracyclines is limited chemically to a single hydroxyl group, a marked difference exists in their spectrum of antitumor activity. Doxorubicin has been more effective than daunorubicin in the treatment of metastatic solid tumors and sarcomas. The cardiac toxicity associated with the chronic administration of both these agents, however, has provided impetus to design a new generation of anthracycline analogues. The long-term assessment of clinical outcome for children successfully treated for malignancy further substantiates the concerns regarding anthracycline-induced cardiotoxicity.¹⁶ None of these anthracycline analogues is devoid of cardiac toxicity, but closely related

molecules may have significant advantages. For example, the anthraquinones (e.g., mitoxantrone) demonstrate less cardiotoxicity and have remission-inducing activity in acute non-lymphocytic leukemia.¹⁷ Thus, in modifying the chemical structure of a natural product in an attempt to enhance its therapeutic selectivity, the synthetic organic chemist plays a critical role in this process of drug development.

Natural product research has yielded other effective antineoplastic drugs. Although most of these agents have been identified in fermentation broths of microbial organisms, plants also have provided a number of active antineoplastic agents. One of the earliest plant-derived drugs resulted from a chance observation. In the 1950s, Nobile and colleagues¹⁸ were investigating interesting plant extracts used by primitive peoples. This attempt to take advantage of tribal medications, primarily natural products, represented an early entree into the discipline known as *ethnopharmacology*.

The leaves of the Jamaican periwinkle plant, *Vinca rosea*, were used to make a tea that was reported to be of benefit in diabetes.¹⁸ During the initial animal investigations, the extract of these leaves was administered orally to both rats and rabbits without any observed effect on blood sugar levels. Subsequent administration of the aqueous extract of the periwinkle plant by injection to rats had a dramatic lethal effect within a week. Postmortem examination of the animals demonstrated that the rats had died of sepsis related to bone marrow suppression. Isolation and chemical characterization of the responsible chemical factors were accomplished using a bioassay-guided approach (i.e., granulocytopenia in the treated animals) for identifying the effective component of this aqueous extract of the plant. The compound was determined to be an organic base and subsequently was called *vincaleukoblastine*. This agent demonstrated carcinostatic activity against both a transplanted murine mammary adenocarcinoma and a rat-transplanted sarcoma.¹⁸ The mechanism of action (i.e., inhibition of microtubule formation) proved to be unique and provided the basis for an entirely new area of research for cancer drug development.

In contrast to using the complicated biologic end point of the peripheral blood granulocyte count from an intact animal, simple and more rapid screens (e.g., molecular target-based or *in vitro* cell cytotoxicity assays) currently are used to guide fractionation of extracts for isolation and characterization of active components. After final chemical identification of the plant-derived chemical antineoplastic entity, validation of antitumor activity in an *in vivo* model is still required. Sufficient supplies of the active agents isolated from natural product sources are needed to conduct adequate *in vivo* confirmatory studies. Adequate supply was a problem with the periwinkle extract in its early development, and it remains problematic for many natural product agents now being isolated.

Several new plant-derived natural products have proven to be of extreme interest in the treatment of cancer. Taxol was isolated from the bark of the Pacific yew tree in 1971,¹⁹ and it has a unique mechanism of antitumor activity that involves stabilization of microtubule assembly with resultant inhibition of the normal dynamics of microtubule formation.²⁰ This agent has a broad spectrum of antitumor activity, and it is active against a number of human tumor xenografts, including breast cancer, ovarian cancer, and other malignancies. Subsequent clinical studies have confirmed the high degree of activity in patients with a wide range of solid tumors, including breast, ovary, head

and neck, esophagus, testes, and lung malignancies.²¹ Initially, a major obstacle to defining the role of Taxol in cancer therapy related to the difficulties encountered with drug supply.¹⁴ Semisynthesis from 10-acetyl baccatin III, was eventually accomplished, and new sources of Taxol from nursery species have been identified. The supply issue has now been fully resolved with the successful total synthesis of this complex molecule.^{22,23} Moreover, advances in the chemistry of isoterines and taxoid anticancer agents have facilitated the synthesis of second-generation taxoid compounds with activity against drug-resistant cancer cells.²⁴ The history of the development of Taxol is important because it highlights the complexities involved with development of any cancer drug—namely challenges in supply of drug, difficulties with synthesis of drug, issues of drug formulation, and obstacles associated with the implementation of successful clinical studies.

Another natural product that has been under investigation for many years, but only now found to have broad activity against various human malignancies, is derived from the bark of *Camptotheca acuminata*, a tree prized for its medicinal properties in traditional Chinese medicine.²⁵ The camptothecin derivatives are unique because they inhibit topoisomerase I, a key enzyme that maintains DNA in a torsionally relaxed state.^{26,27} Both topotecan and CPT-11, which are derivatives of camptothecin, have significant activity in patients with advanced malignancies, including colorectal cancer, esophageal cancer, non-small cell and small cell lung cancer, and cervical cancer.^{28–30} Significant efforts continue to focus on developing novel analogues of camptothecin with enhanced biophysical and biologic activity. 9-Nitroaminocamptothecin and 9-aminocamptothecin are currently in advanced stages of clinical testing.³¹

Marine organisms represent a largely unexplored and untapped source of unique toxic chemicals. These toxins are elaborated by sponges and other sessile saltwater organisms as defenses against their predators. Several highly potent agents demonstrate interesting antitumor activity against unique molecular targets in preclinical models, and some examples include the bryostatins (which inhibit protein kinase C), the dolastatins and halichondrins (which bind to microtubules), and the tunicate-derived ecteinascidins (which bind in the minor groove of DNA).^{31,42–46} Although the marine environment represents an untapped potential source for interesting new chemical entities, certain unique problems affect this biosphere. Scale-up procurement of bulk material from marine sources presents a special challenge in biomass collection. The potency of many of these agents may ameliorate this supply problem, but selectivity against the tumor (and not the normal host) must first be demonstrated. In addition, the highly potent natural products present additional challenges for clinical investigators conducting phase I studies. For example, clinical pharmacologic studies may be impossible if the active species is present in such low levels that detection by even very sensitive analytic methods is not feasible at clinically tolerated doses. The rich diversity of chemical structures found in nature provides the impetus for continued research in this area. Moreover, the use of combinatorial chemistry technology may be incorporated into the process once novel therapeutic leads are identified from these natural products.

Cancer drug discovery may also result from a totally fortuitous experimental observation. The discovery of platinum complexes as antiproliferative agents with remarkable clinical

activity demonstrates the importance of enlightened empiricism combined with dogged persistence in clinical testing and development. In 1965, Rosenberg³⁷ observed that an electric current passing through platinum electrodes could inhibit *Escherichia coli* bacterial cell division. This discovery was confirmed by the subsequent testing of platinum complexes in murine tumor model systems. Cisplatin inhibited the development of sarcomas, and other platinum complexes also were found to be effective in the preclinical models. The early clinical trials demonstrated antitumor activity in patients with advanced malignancies, but the excessive initial toxicity (nephrotoxicity) raised serious concern among the clinical investigators. The demonstration that adequate hydration and slow infusion reduce the degree of renal toxicity permitted further evaluation of the agent. The responses observed in testicular cancer and ovarian cancer led to approval of the drug approximately 6 years after the initial clinical trial. This excellent anticancer drug might have been discarded in error without the foresight of both preclinical and clinical investigators who were convinced of the drug's potential, were committed to the systematic testing of the drug, and were clever enough to find ways to deal with its toxicity. Most of the antineoplastic agents have been discovered through empiric screening efforts or represent chemical modifications of lead compounds discovered in cancer screenings.

Screening methods can either be simple, such as a well-characterized cell line or a defined enzymatic target, or complex, such as an *in vivo* animal tumor. In general, current efforts favor simple systems that accommodate high volumes of unknown compounds. The end point of the cancer screen may be a biologic target (e.g., tumor cell cytotoxicity, growth inhibition, differentiation) or a biochemical-molecular target that is known to be important for the survival of cancer cells. The advantages and disadvantages of each of these approaches is presented in Table 19.2-1. Both the cell line and molecular approach may be combined through the use of genetically engineered cell lines that express a specific molecular target.

The evolution of strategies at the National Cancer Institute (NCI) illustrates the changes in screening that have resulted from the advances in cancer biology and cancer genetics. The early NCI cancer screening efforts used murine leukemias (L1210 and P388) as the index tumors in an *in vivo* screening effort.³⁸ The screen identified agents that had efficacy in humans in the treatment of leukemias and lymphoproliferative malignancies, such as hydroxyurea and the nitrosoureas. However, the failure of this screen to identify active drugs for the major solid tumors resulted in a significant change in 1975 in the approach of the NCI when animal solid tumor and human tumor xenografts were added as a secondary *in vivo* tumor panel.³⁸ In 1985, a second major change was made.^{38,39} The increasing availability of a growing number of cell lines derived from human solid tumors and well characterized with respect to drug response patterns, growth factor dependence, oncogene expression, and other biochemical and molecular features presented an opportunity to focus screening efforts on the unique biology of human solid tumors.

A number of human solid tumor cell lines were selected to provide a disease-oriented approach to drug discovery in contrast with the previous compound-oriented drug discovery methods. A total of 60 human tumor cell lines derived from seven cancer types (e.g., lung, colon, melanoma, kidney, ovary, brain,

TABLE 19.2-1. Comparison of Cancer Screening Devices

Screen	Advantages	Disadvantages
Tumor cell line-based assays	High-volume assay Has identified many current cancer drugs Defines agent with effect on tumor cell and displays the pattern of cellular response Defines agents that cross cell membrane and withstand the intracellular milieu	Mechanism of action not defined by this approach May define agents that are nonspecifically toxic to cells Does not elucidate the cellular target responsible for the observed effect
Mechanistic or molecular targeted assays	High-volume assay Has a rational basis for drug discovery May provide agents specifically aimed at a critical point in the tumor cell Potential selective antitumor activity May provide novel classes of antitumor agents	Despite scientific appeal, approach only more recently implemented No guarantee that agents will enter the cell or withstand intracellular milieu No guarantee that agents will be selective

leukemia) formed the original cell line panel. Breast cancer cell lines were subsequently added. The initial concept proposed that leads demonstrating disease specificity would be identified, and activity could be further examined by *in vivo* testing in nude mice, using the most sensitive *in vivo* index tumor cell lines.

In the current NCI anticancer screen, each candidate agent is tested over a broad concentration range against every cell line in the panel.^{3,4,38-40} Active compounds are selected for further testing based on several different criteria: disease-type specificity in the *in vitro* assay, unique structure, potency, and demonstration of a unique pattern of cellular cytotoxicity or cytostasis, indicating a unique mechanism of action or intracellular target. The agents selected for further investigation are then subjected to additional testing to assess their *in vivo* therapeutic index.^{3,41} The current version of the cancer drug screening program of the NCI has been in operation since 1990, and a number of novel chemical entities have been identified for further evaluation. This high-capacity screen was designed to accommodate approximately 10,000 individual chemicals tested annually, with additional capacity for screening natural product extracts. Approximately 5% of the compounds tested in the initial screen show sufficient activity to warrant further evaluation in *in vivo* screens or biochemical-molecular assays. More than 60,000 agents have been screened against a panel of 60 human cancer cell lines. The tumors that are represented in this cell line panel include melanomas; leukemias; and cancers of the breast, prostate, colon, ovary, kidney, and central nervous system. To date, this approach has identified five novel agents (e.g., a tyrosine kinase inhibitor, a protein kinase C inhibitor, and several disease-specific agents) for further testing in clinical trials. It is hoped that an overall assessment of the clinical usefulness of this novel cell line screening approach will be feasible in the near future.

The concept of cancer drug discovery that is based on high-volume screens, whether oriented toward a cell line-based or molecular target, relies on the acquisition of a large source of diverse materials for examination. In the case of the NCI, an extensive program for acquiring both defined chemical entities and diverse natural products has been pursued. Enormous effort was initially invested to standardize the *in vitro* assay, and sufficient time should be provided to fully evaluate the clinical utility of its early findings.

The pharmaceutical industry is also actively engaged in the procurement of large numbers of interesting chemical structures and natural products for testing in their respective cancer screens, many of which focus on specific molecular targets. Difficult decisions must be made to choose among the large number of unknown entities for initial testing and to aid in the prioritization of known active compounds for further development. Computer programs have been developed to assist in this prioritization and to enhance the diversity of potential chemical entries and crude natural products introduced into the screening process.^{40,42} For example, there are significant challenges in representing, analyzing, and storing the vast amount of experimental data generated by the substances tested in the *in vitro* screen of the NCI. In Figure 19.2-3, drug testing data are represented as a mean graph presenting growth inhibition in a standard bar graph.³⁸ The mean graph is constructed by projecting bars to the right or left of the mean, depending on whether an individual cell line is more or less sensitive than the average line in the panel. Furthermore, the length of each bar is proportional to the relative sensitivity of the cell lines. Thus, each agent can be represented by a characteristic fingerprint of cell line responsiveness.

After an agent has been tested in the cancer screen, its unique response pattern can be compared with the results from all other agents within the database. A computer program called COMPARE uses a simple algorithm for aligning and contrasting the patterns for each compound with the patterns of other compounds in the database.^{43,44} A compound is entered into the program as a seed, and the computer database elicits a list of those agents that have similar patterns of tumor cellular responsiveness. In Table 19.2-2, an example is presented for the introduction of a seed compound and the resulting list of agents that had similar patterns of cellular cytotoxicity. A correlation coefficient is also expressed, relating the closeness of the seed to those agents listed by the computer program. Close correlations between agents appear to have biologic and pharmacologic importance, implying a common intracellular target despite a dissimilarity in structure (e.g., tubulin-binding agents, topoisomerase-interactive agents). The COMPARE program has several important features.⁴⁴ It can identify the intracellular target or mechanism of action of a new compound through a comparison of its fingerprint with known agents. It

ADRIAMYCIN

RUBIDAZONE

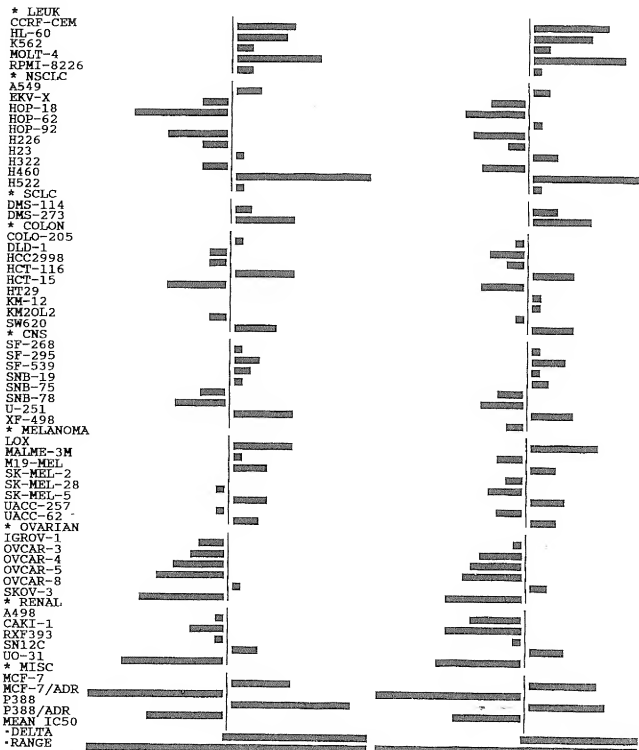


FIGURE 19.2-3. Mean graph representation of antitumor effects in the National Cancer Institute's (NCI) cancer screen. The effects of a specific agent on the tumor cell lines in the NCI cancer screen are shown by the construction of a mean graph presentation of the data. Screening results for two anthracyclines are charted. A mean concentration of the agent that produces the same level of response for all the cell lines in the screen forms an anchor point for this graphic presentation. The individual response of each cell line to the agent is then depicted by a bar graph extending to the right or left of the mean, depending on whether the cell line was either more or less sensitive than the average response, respectively. The length of each bar is proportional to the relative sensitivity when compared with the mean determination. CNS, central nervous system; Leuk, leukemia; NSCLC, non-small cell lung carcinoma; Misc, miscellaneous; SCLC, small cell lung carcinoma.

TABLE 19.2.2. The COMPARE Program*

NSC Number	Parent = Taxol		Chemical Name
	High Concentration	Correlation Coefficient	
152858	1.00E-09	0.812	Maytansine
49842	2.50E-06	0.767	Vinblastine sulfate
332598	5.00E-06	0.745	Rhizoxin
609395	1.00E-08	0.703	Halichondrin B
757	6.25E-04	0.691	Colchicine
67574	1.00E-03	0.628	Vincristine sulfate
376125	1.00E-08	0.628	Dolastatin

*An extensive database has been generated from the many agents tested in the National Cancer Institute's cancer screen. The unique patterns of cellular response can be used to characterize an agent by its "fingerprint" of cytostasis or cytotoxicity. Agents that have common cellular targets can be identified by the use of the computerized database. For example, introduction of a seed or parent compound into the COMPARE program elicits a list of those agents that exhibit similar patterns of tumor cell response. In the example in this table, the agents listed are known to be tubulin-interactive agents. The correlation coefficient for the relation of each agent to the seed or parent compound is provided. High concentration refers to the highest concentration of the agent used during the screening experiment. NSC Number refers to the individual identification number assigned to each agent submitted to the National Cancer Institute.

can search for compounds previously tested in the cancer screen that have a fingerprint similar to that of a lead compound known to inhibit a unique target. It also has the power to detect inhibition of integrated biochemical and molecular pathways that are not adequately represented by a single molecule or molecular interaction. The comparison also allows recognition of a new agent that does not match with compounds of known mechanisms of action. Given the critical roles of an intact cell-cycle checkpoint and apoptotic pathways in determining chemosensitivity, it is clear that such an algorithmic approach may help identify candidate anticancer drugs that are not dependent on an intact checkpoint and apoptosis function. Finally, this strategy provides the rational basis for future pharmacophore development.

Computer approaches to data analysis similar to that described by the NCI are being developed by industry to search for agents interacting with specific molecular targets. In addition, the NCI has conducted an elaborate characterization of specific molecular targets expressed by the existing tumor cell lines within its screen. For example, because certain cell lines are known to contain a mutated or overexpressed oncogene, such as *k-ras* or *HER-2/neu*, it is possible to search the existing database for agents active against only those particular cell lines. This process may ultimately combine the advantages of both cell line-based and molecular screens, but will require separate validation to confirm that identified leads do indeed interact with the purported molecular target in specific assays directed at that entity. Although the NCI cell line screen represents a carefully constructed system for obtaining and analyzing voluminous data on diverse compounds, alternative screening systems in academic centers and industry increasingly rely on high throughput assays based on specific molecular targets, against which combinatorial chemistry inventories are tested. A good example of this approach is the potent anti-mitotic agent monastrol, isolated by Mayer et al.⁴⁵ This agent

targets kinesin Eg5, a mitotic protein required for spindle bipolarity and, thus, acts to inhibit the process of mitosis.

MOLECULAR-TARGETED SCREENING

From a scientific perspective, a compelling argument can be made for focusing on a well-defined molecular target and for using computer-based approaches to design small molecules that would specifically interact with this target.

With the rapid advances being made in defining the molecular pathology of neoplastic cells, specific oncogenes have been identified that are expressed uniquely in malignant tissue. The discovery of inappropriately expressed or mutated genes has provided an impetus for the establishment of numerous screens designed to detect specific inhibitors or modulators of the products of these abnormal genes. Intracellular signaling pathways that mediate the actions of growth factors and oncogenes on cell proliferation, such as protein kinases, G proteins, and transcription activators, provide additional novel targets for anticancer drugs. However, given the considerable overlap of access to various growth-factor signaling pathways (many signals use the same distal steps), signal transduction inhibitors may lack specificity for the neoplastic cells.⁴⁶

As an alternative to targeting these intracellular pathways, significant attention has focused on strategies to inhibit the process of angiogenesis.⁴⁷ This concept stems from the seminal work of Folkman and colleagues⁴² who proposed that the growth of a tumor mass is dependent on the formation of a vascular network that supplies the tumor with essential nutrients. The targeting of the tumor vasculature has two potential advantages over conventional biochemical and molecular targets. The first is that this approach does not require tailoring of therapy to the unique genetic makeup of the tumor; because it appears that all solid tumors are dependent, to some extent, on angiogenesis for growth. In addition, the target of this approach is the normal

vascular endothelial cells that are genetically stable and, thus, less likely to become drug-resistant.

Advocates for the use of mechanistic-based approaches to novel drug discovery have emphasized the potential for selectivity that may result from the use of molecular targeting.^{7,48-50} The expression of identical or closely related molecular or biochemical targets in normal tissue must always be considered. Mutant oncogenes and their corresponding protein products appear to be the most attractive targets for drug design. Two examples include the fusion protein that results from the BCR-ABL translocation in chronic myelogenous leukemia (CML) and the interference with tumor suppression resulting from the binding of papillomavirus proteins to the RB (retinoblastoma) gene in cervical carcinoma.

In CML and in approximately 20% of adult patients with acute lymphocytic leukemia (ALL), a characteristic reciprocal translocation between chromosomes 9 and 22 is observed. The protooncogene (ABL) from chromosome 9 is translocated at the breakpoint cluster region (BCR) on chromosome 22. This translocation encodes the Bcr-Abl protein, which expresses constitutively activated tyrosine kinase function. It is a 210-kD oncoprotein, and expression of p210 BCR-Abl induces a disease in mice resembling CML, confirming the critical role of this oncoprotein in the development of CML.^{51,52} The p210 Bcr-Abl protein is present in 95% of patients with CML and in 5% to 10% of adults with ALL for whom there is no evidence of CML. A second fusion protein of 185 kD is found in 10% of adult cases and 5% to 10% of pediatric cases of ALL, but not in CML.

It is clear that expression of this genetic rearrangement is essential for maintaining the malignant phenotype.⁵³ In addition, transfection of the specific DNA for the BCR-ABL-encoded protein kinase into the hematopoietic stem cells of mice results in the induction of a malignant disorder *in vivo* with similarities to the clinical illness in humans.⁵⁴ Modification of these murine models could provide a potential opportunity to test promising new therapeutic products *in vivo*. Moreover, the aberrant tyrosine kinase resulting from these abnormal genetic rearrangements (BCR-ABL) within the hematopoietic stem cells does not exist in normal host cells. This abnormal gene provides an ideal molecular target for therapeutic intervention. The crystal structure of several protein kinases has been solved, and a number of compounds have been designed based on the structure of the adenosine triphosphate (ATP) binding site or the active site of the enzyme. In screening against the recombinant BCR-ABL kinase protein, the 2-phenylaminopyrimidine derivative known as CGP 57148 (STI 571) proved to be a potent and selective inhibitor, targeting the ATP binding pocket.⁵⁵ This compound inhibits all ABL tyrosine kinases at submicromolar concentrations *in vitro*, and it has minimal to no inhibitory effect on the colony-forming potential of normal bone marrow cells. CGP 57148 appears to be selectively toxic to cells expressing the BCR-ABL tyrosine kinase.^{56,57} A phase I clinical trial has been completed in CML patients who were unsuccessful with interferon therapy.^{58,59} CGP 57148 was given orally on a daily basis, and treatment was well tolerated, with the most common toxicities being only mild nausea (grade 1), muscle cramps, and arthralgias. With regard to its clinical activity, significant hematologic responses have been observed, with 100% clinical complete response at daily doses greater than 300 mg and a 40% to 50% cytogenetic response. Clinical investigations are in

progress to validate the clinical efficacy of this novel agent. Studies have shown that the drug also has potent activity against the platelet-derived growth factor receptor, and in experimental studies, it inhibits tumors that overexpress this receptor. Thus, this compound may have broader application than just for CML.

Another potential target for therapeutic intervention has evolved from an enhanced understanding of the role of aberrant tumor suppressor protein function in the malignant process. It is appreciated that more than 80% of cervical carcinomas have evidence of integrated DNA sequences from papillomaviruses.⁶⁰ Human papillomavirus-16 has been implicated frequently as a causal role of this malignancy. Extensive molecular investigation of the association of human papillomavirus with cervical carcinoma has identified specific nuclear proteins that interact with the tumor suppressor gene RB.⁶¹ In fact, the complex protein-protein interaction between the E7 protein from human papillomavirus-16 and the retinoblastoma suppressor protein (pRB) is believed to be important in the cellular transformation that leads to cervical carcinoma. Expression of the E7 protein apparently occurs both within cells from patients with cervical carcinoma and from cell lines derived from this malignancy. The inactivation of the RB tumor suppressor gene by this protein appears to be reversible. There is significant interest in identifying agents that could selectively interfere with this deleterious E7-RB interaction.

DRUG DEVELOPMENT

There is an urgent need to move promising new therapies into clinical trials. However, important and clinically relevant information may be lost by proceeding immediately from a primary *in vitro* screen to a clinical trial without defining the *in vivo* activity of an agent, its pharmacokinetics and schedule dependency in animals, and its profile of toxicity for normal and malignant cells and tissues. Each of these issues is important and must be addressed in a timely manner to provide safe and reasonable starting doses for implementing phase I trials in patients. The steps required in the development of a cancer agent for clinical practice are complex, and as outlined in Figure 19.2-4, they are both time- and resource-intensive.

Secondary *in vivo* studies to optimize the exposure time to an agent and to define mechanisms of resistance are useful for the investigators planning *in vivo* studies. Examination of the dose response data for several tumor cell lines should permit a selection of the optimal tumor system for subsequently evaluating *in vivo* efficacy. Furthermore, preliminary pharmacologic studies in non-tumor-bearing animals provide useful information about the plasma concentrations achievable and an estimate of the acute toxicity after systemic administration of a new agent. Success in identifying new therapies relies on the expeditious, yet careful, conduct of those studies pertinent to developing a promising *in vivo* observation (derived from either the cell line screen or the molecular models) into an actual drug candidate.^{59,62}

IN VIVO ANTITUMOR ASSAYS CURRENTLY IN USE

In the current NCI development schema, the human tumor cell line most sensitive to an active candidate *in vitro* is selected

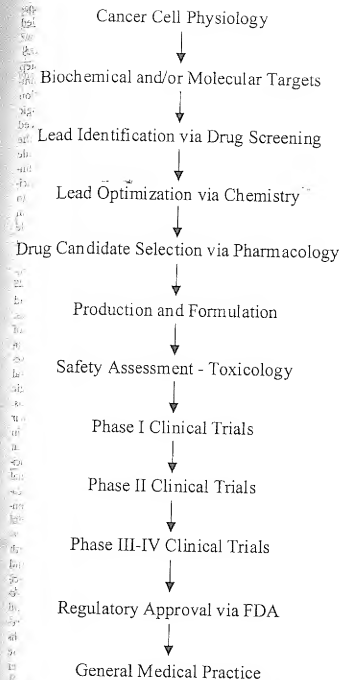


FIGURE 19.2-4. Steps in cancer drug development. FDA, U.S. Food and Drug Administration.

for testing as a xenograft in a subcutaneous implant site in a nude mouse. Compounds identified in molecular screens are usually tested against human or murine tumors engineered to overexpress the specific drug target.

⁶⁵ Failure to demonstrate *in vivo* efficacy for agents that display strong *in vitro* evidence of antitumor activity should prompt additional studies to determine whether a pharmacokinetic or metabolic explanation exists for the loss of activity. The initial lead, either discovered by an empiric screen or as a result of rational chemical design, is usually not the optimal

chemical entity for clinical investigation. Lead optimization and an iterative process between chemists and tumor biologists may be required to enhance the *in vivo* therapeutic index. Factors such as poor solubility and rapid *in vivo* metabolism may be corrected by analogue development. More potent and less toxic derivatives can often be subsequently developed (i.e., provided the molecule is amenable to modification).

PRECLINICAL PHARMACOLOGY

Preclinical studies in mice, rats, and dogs provide essential information about pharmacokinetics and provide a basis for rational schedule development for the new drugs in humans. Factors such as bioavailability (for agents administered by the oral route), metabolism, renal excretion, and penetration into the central nervous system contribute to the understanding of how best to test a new drug in humans. Although there is no guarantee that human subjects will handle a new drug in the same way as the animal species, in most instances the major pathways for drug metabolism and excretion are qualitatively, if not quantitatively, the same across species.

Pharmacokinetic information in animals can also provide a rational basis for dose escalation in humans. Collins and associates⁶⁶ have hypothesized that dose-limiting toxicity in mice and humans is a function of drug exposure, as measured by the area under the drug concentration in plasma \times time curve ($C \times T$). They predict that animals and humans encounter dose-limiting toxicity at the same $C \times T$ for any given drug and that the experimentally determined dose-limiting $C \times T$ can be used as a target for dose escalation in humans. An analysis of experience with phase I drug trials suggested that for most, but not all, drugs, the relationship of $C \times T$ to toxicity holds across species. This work potentially allows the clinical investigator to base initial dose escalation steps on measurements of $C \times T$. Dose escalation can proceed in a more rapid fashion than formerly possible using empiric schemes, and wasteful multiple steps in dose escalation can be avoided. This approach, although apparently valid in retrospective studies, still requires broader validation in a prospective manner.

Drugs that demonstrate substantial interspecies variation in patterns of target tissue activation are not good candidates for this approach. For example, drugs activated by deoxycytidine kinase, such as fludarabine phosphate, are much more toxic to human marrow cells than to mouse bone marrow, presumably as a result of the higher levels of this activating enzyme in human cells.⁶⁷ In this instance, toxicity in humans would not be accurately predicted by the $C \times T$ approach. Furthermore, drug candidates that are excessively potent (e.g., several of the marine natural products) may have biologic effects at plasma concentrations lower than the level of reproducible detection. Consequently, such agents are not acceptable candidates for pharmacologically guided dose escalation.

FORMULATION STUDIES

Although the preliminary pharmacologic and toxicologic studies may begin before a decision on the final formulation of a product, the Investigational New Drug (IND)-directed toxicology should be performed with the final formulation. In addition, other critical studies may be influenced by the formulation (e.g., bioavailability of an oral formulation, insol-

ability of an agent demonstrating interesting antitumor activity in the cancer screen). Three important factors that have an impact on formulation studies include solubility, stability, and dosage requirements.⁶⁸

Because the route of drug administration for antineoplastic agents has primarily been through the intravenous approach, the issue of solubility has provided a substantial challenge for a number of agents with limited aqueous solubility. Efforts to improve the solubility of an agent have primarily involved physical measures, including the use of various mixed solvent systems. Novel approaches, including the use of micronization, liposomal encapsulation, and other unique delivery systems (e.g., cyclodextrins and coacervate systems), have been investigated in an effort to improve methods of drug delivery to tissues. Major efforts are needed to expand the vehicles that are available for intravenous drug delivery of agents with limited aqueous solubility and stability.

The prodrug approach uses chemical modification to solve the difficulty associated with drug insolubility. The most recent example of a simple prodrug approach is the synthesis of the monophosphate of 2-fluoro-adenine arabinoside (fludarabine).⁶⁹ In essence, the halogenated nucleoside was poorly soluble in aqueous solution. In contrast, the monophosphate (fludarabine) was more soluble and readily cleaved enzymatically *in vivo* to the 2-fluoro-adenine arabinoside. The nucleoside is rapidly rephosphorylated after transport to the intracellular compartment and, thus, can be effective as an anticancer agent.

Unique opportunities exist to use monoclonal antibodies to selectively deliver antineoplastic agents to targeted tumor cells. New methods of prodrug administration (e.g., ADEPT) are being evaluated that couple the administration of an anthracycline glucuronide and the use of a human β -glucuronidase conjugated to a monoclonal antibody for selected delivery to a tumor-bearing animal. It is hoped that this novel approach will enhance the selectivity of anticancer agents, and it may have particular utility in the case of highly potent compounds.

TOXICOLOGIC INVESTIGATION

Preclinical toxicology is frequently the final step in the progression of a new chemotherapeutic drug from discovery to initial phase I testing in humans (see Fig. 19.2-4). The major objectives of the preclinical toxicologic studies include (1) the definition of the qualitative and quantitative organ toxicities (including dose and schedule dependencies), (2) the reversibility of these effects, and (3) the initial safe starting dose proposed for humans. In general, the ideal approach is to ensure that the preclinical toxicologic studies accurately reflect the intended clinical investigations in humans (i.e., identical formulation, schedules, and routes of drug administration, and dose levels anticipated to reflect the likely experience in patients).

The protocols for performing the preclinical toxicology at the NCI have changed dramatically since the late 1970s.^{70,71} Numerous schedules of drug administration were examined in a variety of animal species in the era before 1980. The emphasis later focused on mouse lethality studies for the initial dose-range-finding studies [i.e., lethal dose in 10% of mice (LD_{10}), LD_{50} , and LD_{90}]. The subsequent toxicologic studies were performed on fixed schedules to refine the doses associated with lethal and nonlethal toxicities. The preclinical toxicities reported correlated reasonably well with the subsequent clinical

observations.^{70,72-74} However, the extent of useless information relating to highly lethal murine doses (LD_{50} and LD_{90}) led to a redesign of the toxicologic studies.

The current toxicologic investigations accepted by the U.S. Food and Drug Administration involve a simplified two-step approach. The initial step focuses on acute toxicity in small animals (e.g., mice), and the major end point is a determination of the LD_{10} level. The second phase of preclinical toxicologic investigation is more extensive. In this case, emphasis is placed on a careful qualitative and quantitative characterization of the organ-specific toxicities in rodents associated with the schedule and route of administration that is to be used in the initial clinical trial. Attention is given to defining accurately those toxicities that are likely to be observed at doses slightly higher than the highest nontoxic dose. Careful investigation of the doses in the animals that approximate the highest projected tolerable dose in the model should provide data that are more relevant to the anticipated clinical experience in patients.

In the past, most new antineoplastic agents were tested clinically on two relatively fixed schedules of drug administration—that is, single-bolus intravenous dose once every 3 to 4 weeks, and 5 consecutive days of treatment repeated at 3- to 4-week intervals. The most frequently used toxicologic protocols reflect each of these schedules. Some newer agents entering preclinical evaluation for cancer therapy are being proposed for weekly intravenous administration, continuous intravenous infusion, or oral dosing. It is critically important that the preclinical toxicologic protocol simulates the planned therapeutic approach in patients.

Because substantial variation may exist between species in their tolerance to a given drug, the safety of a projected starting dose in humans is confirmed by examining the preclinical toxicities in at least two species. Both the qualitative and the quantitative toxicities are usually well defined after investigation of a small animal model (e.g., mouse) and a larger animal (e.g., dog). Only occasionally is testing needed in an additional large animal (e.g., monkey), although this species has been shown to be especially useful for defining central nervous system pharmacokinetics.

Certain organ-specific toxicities are reliably detected with the current toxicologic models (e.g., myelosuppression and gastrointestinal toxicity). In contrast, hepatic and renal toxicities are often missed or falsely positive in animal testing. Toxicities involving the heart, lung, nervous system, pancreas, and integument are even less reliably appreciated. At best, the preclinical evaluation can establish a safe starting dose for humans and predict acute organ toxicity. A complete definition of the toxicologic profile of a new agent usually emerges only after extensive clinical experimentation.

CONCLUSION

The discovery and development of novel anticancer agents involves substantial time, effort, and resources. The strategies used for drug discovery range from empiric screening (the source of most of the current active drugs) to rational drug design based on an enhanced understanding of the various biochemical and molecular targets. As outlined in this chapter, an extensive series of preclinical investigations are necessary before the decision to enter clinical trials is made. Significant efforts are then required for the successful completion of clinical studies, in which an individual agent is taken from the ini-

TABLE 19.2-3. Stages in Clinical Testing of New Anticancer Agents

Stage of Drug Testing	Objectives	Patient Population Studied
Phase I	Determine tolerance Maximally tolerable dose Limiting toxicity Reversibility of toxicity Proper schedule Pharmacology Bioavailability Plasma clearance Biotransformation Excretion Therapeutic effect Secondary	Histologically confirmed advanced malignancies; no longer amenable to conventional therapy; physiologically well compensated.
Phase II	Therapeutic effect Determine effectiveness in a panel of human tumors Dose-response relationships Nontherapeutic effects Toxicity in relationship to therapeutic effect	Histologically confirmed advanced malignancy; measurable tumor masses; no longer amenable to conventional therapy; a variety of tumor types in groups of 15 to 30; physiologically well compensated.
Phase III	Therapeutic effectiveness Compare experimental therapy to existing standard therapy Nontherapeutic effects Are toxic effects tolerable in the context of observed therapeutic effect and in comparison with standard therapy?	Histologically confirmed malignancy; patient sample must be of adequate size and uniformity; usually previously untreated; controls usually are selected randomly, but on occasion, historical controls are used.
Phase IV	Therapeutic effectiveness Integration of drug therapy into primary treatment in combination with surgery or radiation therapy (e.g., postoperative drug treatment in breast cancer) Compare to concurrent standard program Nontherapeutic effects Are toxic effects sufficiently minimal to risk giving drug to patients whose tumor might not necessarily recur? Long-term toxic effects require monitoring (second tumors, sterility, marrow aplasia)	Histologically confirmed malignancy; patient sample must be of adequate size and uniformity; controls usually randomized.

dial phase I testing through to the randomized phase III and IV settings (Table 19.2-3). The effective development of new cancer agents demands the close cooperation of a multidisciplinary team that includes basic research scientists, clinical pharmacologists, clinical research nurses, data managers, and clinical investigators. The combined resources of government, academic centers, and the pharmaceutical industry are needed for successfully dealing with the formidable task of identifying effective new therapeutic agents for cancer patients.

REFERENCES

- Kushman D, Chabner BA. Clinical strategies for cancer treatment: the role of drugs. In: Chabner BA, Longo DL, eds. *Cancer chemotherapy and biophysics*. New York: Lippincott-Raven, 1996:1.
- Greiner MR. Cancer drug screening. In: Schilke RL, ed. *Principles of antineoplastic drug development and pharmacology*. New York: Marcel Dekker Inc, 1996:1.
- Greiner MR, Schilke RL, Chabner BA. The National Cancer Institute: cancer drug discovery and development programs. *Semin Oncol* 1992;19:622.
- Farber S, Diamond LA, Metter RD, Sylvester RJ, Wolf JA. Temporary remissions in acute leukemia in children produced by folic acid antagonist, 4-aminopteroyl-glutamic acid (aminopterin). *N Engl J Med* 1948;238:787.
- Kleinberger L, Chomshatz NK, Dornberger P, et al. Fluorinated pyrimidines. A new class of antineoplastic compounds. *Nature* 1967;170:563.
- Enckson J, Neldron DJ, VanDrie J, et al. Design, activity, and 2.8 Å crystal structure of a C2 symmetric inhibitor complexed to HIV-1 protease. *Science* 1990;249:627.
- Johnson RG. Screening methods in antineoplastic drug discovery. *J Biol Chem* 1992;267:1082.
- Schiller M, Newman DJ, Snider K. Discovery and development of antineoplastic agents from natural sources. *Biochem Soc Rev* 1989;3:131.
- Saizawa SE, Liao KS, Felder S, et al. One head, one chemical compound: use of the selective process for anticancer drug discovery. *Asia Oncol* 1994;15:127.
- Liao KS, Wu J, Liao Q. Identification and characterization of a novel synthetic peptide substrate specific for Src-family protein kinases. *Int J Pept Protein Res* 1996;49:587.
- Amel LM. Structure-based drug design. *Curr Opin Biotechnol* 1993;9:356.
- Laughlin JF, Lebl M. New methods in combinatorial chemistry—robotics and parallel synthesis. *Curr Opin Chem Biol* 1997;1:67.
- Spaulier MB, Burger MT, Forde M, Bartlett PA. Synthetic strategies in combinatorial chemistry. *Curr Opin Chem Biol* 1997;1:47.
- Crigg GM, Newman DJ, Woss RB. Coral reefs, fisheries and thermal vents: the worldwide exploration of nature for novel anticancer agents. *Semin Oncol* 1997;24:156.
- Weiss RB, Sarogy C, Clagett-Carr K, Russo M, Leyland-Jones B. Anthracycline analogs: the past, present, and future. *Cancer Chemother Pharmacol* 1986;18:185.
- Stashenko LJ, Steinberg PC, Rao C. Cardiac failure and dysrhythmias 6–19 years after anthracycline therapy: a review of 15 patients. *Med Pediatr Oncol* 1995;24:352.
- Smith LE. Mitoxantrone (Novantrone): a review of experimental and early clinical studies. *Cancer Treat Res* 1983;10:103.
- Noble RL, Beez CT, Gurns JH. Role of chance observations in chemotherapy. *Vitam Horm* 1968;26:282.
- Winn AC, Toker HL, Wall ME, Guggen P, McPhail AT. Plant anticancer agents. VI. The isolation and structure of Taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. *J Am Chem Soc* 1971;93:2225.
- Schiff PB, Fant J, Horwitz SB. Promotion of microtubule assembly in cells by Taxol. *Nature* 1978;277:665.
- Rommelspach EK, Dornberger BC. Drug therapy: paclitaxel (Taxol). *N Engl J Med* 1996;332:1094.
- Moss J. Steps to a successful synthesis. *Nature* 1994;367:564.
- Gordon M. Taxol supply problem? What problem? *Nat Biotechnol* 1996;14:1625.
- Ojima J, Liu S, Wang F. Recent advances in the medicinal chemistry of taxoids with novel heterocyclic side chains. *Curr Med Chem* 1999;6:527.
- Wall ME, Wall MC, Cook CR, et al. Plant anticancer agents. I. The isolation and structure of camptothecin, a novel alkaloidal leukemia and tumor inhibitor from *Camptotheca acuminata*. *J Am Chem Soc* 1966;88:5885.
- Hsiang VH, Hittelman SN, Hsieh S, Jia LF. Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. *J Biol Chem* 1985;260:14773.
- Hsiang VH, Liu LF. Identification of mammalian DNA topoisomerase as an intracellular target of the anticancer drug camptothecin. *Cancer Res* 1988;48:1722.

28. Rothberg ML, Bluske DC. Topoisomerase I inhibitors in the treatment of colorectal cancer. *Semin Oncol* 1999;26:632.
29. Cunningham D. Setting a new standard—irinotecan (Cimvet) in the second-line therapy of colorectal cancer. Final results of two phase III studies and implications for clinical practice. *Semin Oncol* 1999;28:1.
30. Bown DJ, Sella G, Laidinger P, et al. Phase II trial of weekly irinotecan plus cisplatin in advanced esophageal cancer. *J Clin Oncol* 1999;17:3270.
31. Geronzielli DC, Schijm J, Wolf ME, et al. DNA topoisomerase I-targeted chemotherapy of human colon cancer in xenografts. *Semin Oncol* 1999;26:1016.
32. Fenn G, Herald CL, Doubek DL, et al. Isolation and structure of tyrosinase I. *J Am Chem Soc* 1985;107:6848.
33. Bai R, Patel GR, Hamed E. Dolastatin 10, a potential cytotoxic peptide derived from a marine animal. Inhibition of tubulin polymerization mediated through the v-src allele binding domain. *Biochem Pharmacol* 1996;59:1041.
34. Kai RL, Patel KD, Herald CL, et al. Halichondrin B and homohalichondrin B, unique natural products binding in the v-src domain of tubulin: discovery of tubulin-based mechanisms of action by analysis of differential cytotoxicity drug. *J Biol Chem* 1991;266:15892.
35. Boudreau M, Lavoie E, Fernandez JM, et al. Effect of cyclosporin A on the interaction between DNA binding proteins and DNA. *Anticancer Drug Deliv* 1993;14:179.
36. Kirschner KL. Antitumor compounds from tunicamycin. *Med Res Rev* 2000;20:1.
37. Rosenberg B. Fungitoxin studies with cisplatin. *Cancer* 1986;59:2302.
38. Boyd MR. Some of the NCI preclinical antitumor drug discovery scans. *PTG Update* 1999;33.
39. Sieben F, Sturges R, Sweeney D, et al. New colorimetric cytotoxicity assay for antitumor drug screening. *J Natl Cancer Inst* 1990;82:1107.
40. Chabone JA. In defense of cell-line screening. *J Natl Cancer Inst* 1990;82:1053.
41. Donab J, Bibbs MC. Therapeutic index, a vital component in selection of anticancer agents for clinical trial. *J Natl Cancer Inst* 1989;81:888.
42. Hedges L. Computer-aided selection of compounds for antitumor screening: validation of a statistical/chemometric method. *J Chem Inf Comput Sci* 1981;21:121.
43. Patel KD, Stromacker RH, Hedges L, et al. Display and analysis of patterns of differential activity of drugs against human tumor cell lines: development of mean graph and COM-PAKE algorithms. *J Natl Cancer Inst* 1990;81:1098.
44. Weinstein JN, Myers TG, O'Connor PM, et al. An information-intensive approach to the molecular pharmacology of cancer. *Semin Oncol* 1997;24:343.
45. Myers TG, Rapana TM, Huggers SJ, et al. Small molecule inhibitors of mitotic spindle instability identified in a pharmacologic screen. *Semin Oncol* 1999;26:971.
46. Gibbs JR. Anticancer drug targets: growth factors and growth factor signaling. *J Clin Invest* 2000;105:9.
47. Keeble E, Ben-Sasson SA. Anticancer drug targets: approaching targetogenesis. *J Clin Invest* 1999;104:1497.
48. Haasdonk D, Folkman D. Fungitoxin and emerging mechanisms of the antitumor activity of this fungitoxin. *Cell* 1986;86:383.
49. Kadon JG. Choosing anticancer drug targets in the postgenomic era. *J Clin Invest* 1999;104:1505.
50. Sellers WK, Thibodeau DE. Apoptosis and cancer drug targeting. *J Clin Invest* 1999;104:1555.
51. Shapiro GL, Harper JW. Anticancer drug targets: cell cycle and checkpoint control. *J Clin Invest* 1999;104:1616.
52. Kelliker M, Knox A, McLaughlin J, Witte ON, Rosenberg N. Differences in oncogenic potency but not target cell specificity distinguish the two forms of the hereditary oncogene. *Mol Cell Biol* 1993;13:14710.
53. Daley-CQ, Van Etten RA, Baltimore D. Induction of chronic myelogenous leukemia by the P129/321 gene of the Philadelphia chromosome. *Semin Oncol* 1999;24:824.
54. Scagell C, Shorski T, Nicholas NG, et al. Selective inhibition of leukemia cell proliferation by BCR/ABL antisense oligodeoxynucleotides. *Semin Oncol* 1991;23:502.
55. Kelliker M, McLaughlin J, Witte ON, Rosenberg N. Induction of a chronic myelogenous leukemia-like syndrome in mice with v-ABL and BCR/ABL. *Proc Natl Acad Sci U S A* 1990;87:6665.
56. Druker BJ, Taniuchi S, Buchdunger E, et al. Effects of a selective inhibitor of the ABL tyrosine kinase on the growth of BCR/ABL-positive cells. *Nature Med* 1996;2:561.
57. Deininger MW, Goldman JM, Lyman N, Melo JV. The tyrosine kinase inhibitor CGP57148 selectively inhibits the growth of BCR/ABL-positive cells. *Blood* 1997;90:3091.
58. LeGendre F, Moloney L, Chen L, et al. In vivo eradication of human BCR/ABL-positive leukemia cells with an ABL kinase inhibitor. *J Natl Cancer Inst* 1999;91:103.
59. Druker BJ, Sawyers CL, Talpaz M, et al. Phase I trial of a specific ABL tyrosine kinase inhibitor (CGP 57148) in intermediate refractory chronic myelogenous leukemia patients. *Proc Am Soc Clin Oncol* 1996;15:24(abst).
60. Druker BJ, Lydon NB. Lessons learned from the development of an ABL tyrosine kinase inhibitor for chronic myelogenous leukemia. *J Clin Invest* 2000;105:5.
61. van Heston H. Molecular pathogenesis of cancer of the cervix and its connection by specific human papillomavirus types. In: van Heston H, ed. *Human papillomavirus*. Berlin: Springer-Verlag, 1994:135.
62. Jones RF, Wozniak RJ, Patrick DR, et al. Identification of HPV-16 E7 peptides that are potent antagonists of E7 binding to the retinoblastoma suppression protein. *J Biol Chem* 1990;265:12782.
63. Corbett TH, Wozniak A, Gerphel BS, Janda L. In situ and in vivo models for detection of new antitumor drugs. In: Hanja B, Horka L, White RJ, eds. *Proceedings of a workshop at the 14th International Congress of Oncology* at Kyoto, Tokyo, University of Tokyo Press, 1995:6.
64. Grindley GB. Current status of cancer drug development: failure or limited success? *Cancer Cells* 1990;2:163.
65. Beggs AE. The national capsule assay and its use as a drug screening system. In: Hellman K, Carter SK, eds. *Fundamentals of cancer chemotherapy*. New York: McGraw-Hill, 1987:173.
66. Gelfand TH, Velezquez PA, Baker III L. The P-388 murine tumor cell line: a drug screening model. *Proc Natl Acad Sci* 1975;72.
67. Collins JH, Grieshaber CK, Chabone JA. Pharmacologically guided phase I clinical trial based upon preclinical drug development. *J Natl Cancer Inst* 1990;82:121.
68. Debonis JW, Leiby JM, Staudus AE, Grever MR, Malspess L. Altered pharmacokinetics of fluorouracil phosphate in the rat and analysis of a series of fluorouracil derivatives. *Proc Am Soc Cancer Res* 1989;28:1415.
69. Doughty JF, Graddick JC. The formulation of anticancer drugs. In: Hellman K, Carter SK, eds. *Fundamentals of cancer chemotherapy*. New York: McGraw-Hill, 1987:212.
70. Breckman RW, Cheng YC, Schabli FM, Monaghan JA. Metabolism and chemotherapeutic action of 3- β -acetoxy-4-oxo-5- α -cholestanol-20-one (irinotecan) and its metabolite, 3- β -acetoxy-4-oxo-5- α -cholestanol-20-one. *Cancer Res* 1989;49:3671.
71. Grieshaber CK, Mansour S. Relations of preclinical toxicology to findings in early clinical trials. *Cancer Treat Rep* 1986;70:65.
72. Lowe MC, Davis KD. The current regulatory protocol of the National Cancer Institute. In: Hellman K, Carter SK, eds. *Fundamentals of cancer chemotherapy*. New York: McGraw-Hill, 1987:228.
73. Schabli FM, Anderson T. The effect of animal studies in selecting clinical toxicity of cancer chemotherapeutic drugs. *J Clin Pharmacol* 1978;8:222.
74. Lowe M. Large animal toxicological studies of anticancer drugs. In: Hellman K, Carter SK, eds. *Fundamentals of cancer chemotherapy*. New York: McGraw-Hill, 1987:238.
75. Schuring JE, Braden WT. Small animal toxicology of cancer drugs. In: Hellman K, Carter SK, eds. *Fundamentals of cancer chemotherapy*. New York: McGraw-Hill, 1987:248.

SECTION 2

COMBINATORIAL CHEMISTRY

DAVID J. AUSTIN

PRINCIPLES OF COMBINATORIAL CHEMISTRY

REVOLUTION IN SYNTHETIC AND MEDICINAL CHEMISTRY

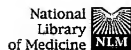
The field of combinatorial chemistry represents a revolution in both the concepts and construction of chemical entities. This revolution has not only changed the fields of chemical catalysis, materials science, and methods development, but also has impacted the field of drug development. The impact of combinatorial chemistry is likely to be as significant to drug development as the polymerase chain reaction was in advancing cloning techniques for molecular biology. The postgenomic era is predicted to

present us with between 50,000 and 150,000 unique genes, each encoding a protein product that is potentially a therapeutic target.¹ Between 1000 and 3000 unique members are predicted to exist within the protein kinase family, which is an important class of therapeutic targets.² Because the average medicinal chemist can synthesize approximately 100 molecules per year, it is difficult to envision the identification of unique inhibitors for thousands of proteins using traditional techniques.

This chapter introduces the field of combinatorial chemistry and describes how it has impacted drug development and how it will likely impact future drug development. The aim of this introduction is to serve as a primer for researchers wishing to incorporate chemical diversity into their research program.

WHAT IS COMBINATORIAL CHEMISTRY AND HOW DOES IT RELATE TO CHEMICAL DIVERSITY?

To properly understand what combinatorial chemistry is, the concept of chemical diversity must first be addressed. If diversity is defined as that which represents all possible permuta-



All Databases PubMed Nucleotide Protein Genome Structure OMIM PMC Journals Bool

Search PubMed for

Limits Preview/Index History Clipboard Details
 Display Abstract Show 20 Sort by Send to

About Entrez

Text Version

Entrez PubMed

Overview
 Help | FAQ
 Tutorial
 New/Noteworthy
 E-Utilities

PubMed Services

Journals Database
 MeSH Database
 Single Citation Matcher
 Batch Citation Matcher
 Clinical Queries
 Special Queries
 LinkOut

My NCBI (Cubby)

Related Resources

Order Documents
 NLM Catalog
 NLM Gateway
 TOXNET
 Consumer Health
 Clinical Alerts
 ClinicalTrials.gov
 PubMed Central

☐ 1: Virus Res. 2001 Jul;76(1):87-102.

Related Articles, Links

ELSEVIER
 FULL-TEXT ARTICLE

Different host-cell shutoff strategies related to the matrix protein lead to persistence of vesicular stomatitis virus mutants on fibroblast cells.

Desforges M, Charron J, Berard S, Beausoleil S, Stojdl DE, Despars G, Laverdiere B, Bell JC, Talbot PJ, Stanners CP, Poliquin L.

Department of Biological Sciences, Universite du Quebec a Montreal, P.O. Box 8888, Station Centre-ville, Quebec, H3C 3P8, Montreal, Canada.

Acute infection of fibroblastic cell lines by the Indiana strain of vesicular stomatitis virus (VSV) usually induces dramatic cytopathic effects and shutoff of cellular gene expression. We have compared a series of independent mutants with differences in shutoff induction and found that M was mutated either in the N-terminus (M(51)R) or C-terminus (V(221)F and S(226)R). Furthermore, only double mutants (M mutation and a ts mutation related or not to M) were able to persist on fibroblast cell lines at 39 degrees C. A more detailed investigation of the infection was performed for the mutants T1026, TP3 and G31, differing in their host shutoff effects related to M protein. Viral activity in persistently infected mouse L-929 and monkey Vero cell lines was followed by viral proteins detection, RNA synthesis throughout infection and finally detection of infectious particles. All three mutants cause extensive CPE followed by emergence of persistently infected cells on Vero cells. The same thing is seen on L-929 cells except for T1026 which causes little CPE. Taken together, the results form a basis for further studies to clarify how various viral and cellular factors interact in the establishment of a persistent infection by VSV mutants.

PMID: 11376849 [PubMed - indexed for MEDLINE]

Display Abstract Show 20 Sort by Send to

[Write to the Help Desk](#)

The Vesicular Stomatitis Virus Matrix Protein Inhibits Transcription from the Human Beta Interferon Promoter

MAUREEN C. FERRAN† AND JEAN M. LUCAS-LENARD*

Department of Molecular and Cell Biology, University of Connecticut,
Storrs, Connecticut 06269-3125

Received 14 June 1996/Accepted 27 September 1996

In cells infected by wild-type (wt) vesicular stomatitis virus (VSV) Indiana, host transcription is severely inhibited. DNA cotransfection studies have implicated the VSV matrix (M) protein in this process (B. L. Black and D. S. Lyles, *J. Virol.* 66:4058-4064, 1992). The M protein inhibited transcription not only from viral promoters in plasmids but also from the chromosomally integrated human immunodeficiency virus type 1 (HIV-1) provirus promoter (S.-Y. Paik, A. C. Banerjee, G. G. Harmison, C.-J. Chen, and M. Schubert, *J. Virol.* 69:3529-3537, 1995). In this study, we investigated the effect of wt VSV M protein on expression of a reporter gene under control of a cellular promoter (beta-interferon [IFN- β] promoter), using double transient transfections in BHK and COS-1 cells. The cellular IFN- β promoter was as susceptible to the inhibitory effect of the M protein as the viral promoters used previously. Viral proteins N, P, and G had no significant effect on reporter gene expression. The M protein gene from VSV mutant T1026R1, which is defective in host transcription inhibition, was cloned and sequenced, and its effect on reporter gene expression was tested. The mutant M protein had a methionine-to-arginine change at position 51 in the protein sequence and did not inhibit transcription from either the IFN- β promoter or viral promoters. This VSV mutant is a good inducer of IFN, as opposed to the wt virus, which suppresses IFN induction. These results show that the M protein inhibits transcription from cellular as well as viral promoters and that the M protein does not regulate the IFN promoter any differently from viral promoters. While the M protein may play a role in IFN gene regulation, other viral or cellular factors that provide specificity to the induction process must also be involved.

Infection of mammalian cells with wild-type (wt) vesicular stomatitis virus (VSV), Indiana serotype, results in the inhibition of host RNA and protein synthesis (reviewed in reference 40). wt VSV infections in general do not induce interferon (IFN) (22, 39, 41), but certain mutants of this virus, such as T1026R1 (36), are very good inducers of IFN (24, 32). It is thought that the wt virus produces an inhibitor or suppressor of IFN induction and that those VSV mutants that are good inducers of IFN are defective in the gene coding for the suppressor (23). The suppressing phenotype is dominant, for cells coinfecting with IFN-suppressing and -inducing virus do not induce IFN (25, 26).

The matrix (M) protein of VSV is a potent inhibitor of transcription. Using double transient transfection experiments, Black and Lyles (4) showed that M gene expression caused the inhibition of transcription of the cotransfected chloramphenicol acetyltransferase (CAT) reporter gene. Northern blot and nuclear runoff transcription analyses demonstrated a reduction in the level of the vector-encoded reporter mRNAs and transcription of the target gene, respectively. Recently, Paik et al. (29) demonstrated that VSV M protein can inhibit transcription from the chromosomally integrated human immunodeficiency virus type 1 (HIV-1) provirus, suggesting that the effect of M protein is not limited to transcription from plasmids. The data also supported the role of the M protein in shutoff of host cell transcription.

The ability of M protein to inhibit transcription is only one

of the various functions of this multifunctional protein. It plays a role in virus assembly (reviewed in reference 40), causes the disorganization of the cytoskeleton resulting in cytopathic cell rounding (5, 34), and down-regulates viral RNA transcription (8, 11). It also interacts with the viral genomic nucleocapsid and cellular components such as tubulin and the plasma membrane (9, 10, 27, 28, 42). It has been detected both in the cytoplasm and in the nucleus of infected cells (20) and is a phosphoprotein (2).

The M protein appears to be quite cytotoxic. Several laboratories have reported that while the VSV L, G, N, and P genes are expressed in high levels in transfected cells when controlled from a simian virus 40 (SV40) late promoter, it has been difficult to detect expression of the M gene when driven by the same promoter (4). This is believed to occur because M protein inhibits its own transcription as well as cellular transcription (4). This inhibition is not noted when the M gene is driven by the T7 bacterial virus promoter in a system that does not rely on the host transcription machinery (3). Its involvement in cytopathic cell rounding may also contribute to its low level of expression (5).

Until now, the effects of M protein on expression of target genes from only a few viral promoters, including the SV40 early promoter, the cytomegalovirus (CMV) promoter, and the HIV-1 long terminal repeat, have been examined (4, 29). In this study, we determined whether the M protein had the same effect on a cellular promoter, the human IFN- β promoter, as on the aforementioned viral promoters. The IFN promoter was of particular interest because it could possibly have been regulated differently by the M protein, given the role of IFN in antiviral defense.

We also examined the effects of cotransfection of VSV genes N, P, L, and G on reporter gene expression controlled by the IFN and two (SV40 early and CMV) viral promoters. The

* Corresponding author. Mailing address: Department of Molecular and Cell Biology, University of Connecticut, Storrs, CT 06269-3125. Phone: (860) 486-3974. Fax: (860) 486-4331. E-mail: LucasL@UConnVM.UConn.edu.

† Present address: National Institutes of Health, Bethesda, MD 20892-0455.

results were expected to provide information about whether the N, P, L, or G protein had any regulatory effect on the IFN promoter. Finally, we cloned the M gene from the IFN-inducing VSV mutant T1026R1 and studied the effect of its expression on reporter gene transcription from both the IFN and SV40 promoters, using double transient transfections. The data showed that transcription from the cellular IFN promoter, like transcription from viral promoters, was inhibited by the M protein from wt VSV but not by the VSV G, N, and P proteins. On the other hand, the M protein from mutant T1026R1 did not inhibit transcription from either the IFN or SV40 promoter. The role of the M protein in IFN gene regulation is discussed.

MATERIALS AND METHODS

Cell culture, viruses, and infection. BHK cells were grown in Dulbecco's modified essential medium (DME) containing 10% calf serum and 10% tryptose phosphate broth (Difco) on plastic petri dishes at 37°C. COS-1 cells were grown in DME containing 10% fetal bovine serum as described above. The heat-resistant (HR-C) strain of the Indiana serotype of VSV was used as the wt virus. Mutant T1026R1, a temperature-stable revertant of T1026 (36), was supplied by C. P. Scanners. This mutant was originally derived from the Indiana HR strain of VSV by chemical mutagenesis (36).

Plasmids. Plasmid pTWU54 contains the CAT reporter gene regulated by the human IFN- β promoter region and was generously provided by R. Schleimer, Indiana University School of Medicine (see reference 6 for a detailed description of this plasmid). The expression vectors containing the different VSV genes regulated by the SV40 late promoter were previously cloned into pC119 (35) and were generously provided by M. Schubert (National Institutes of Health). They were as follows: pSVGL containing the G gene (30), pSV-VSL1 containing the L gene (31), pKCM1 containing the M gene (33), pS223 containing the N gene (35), and pLH7 containing the P gene (18). pC119 was used as a control in many of the experiments and to keep the amount of DNA per transfection constant. pSV2CAT contains the CAT gene driven by the SV40 early promoter and was the generous gift of M. Sekellick (University of Connecticut). pCEP4/CAT (Invitrogen) contains the CAT reporter gene regulated by the CMV constitutive promoter. Plasmids were prepared by using Qagen Maxi Prep kit according to the manufacturer's instructions. DNA was quantitated by Hoechst staining.

Cloning and sequencing of the T1026R1 M gene. Confluent cultures of BHK-21 cells grown on 100-mm-diameter plates were infected with T1026R1 at a multiplicity of 10 for 6 h. Cytoplasmic RNA was isolated by standard techniques (21).

The M gene of mutant T1026R1 was amplified by reverse transcription-PCR (RT-PCR) in a single tube (Perkin Elmer). The synthesis of the first single-stranded DNA copy of the M mRNA was initiated by using primer A (5' CCGTCGGAG(TT)₃CATAGG 3') consisting of oligo(TT)₃ flanked at its 3' end with six nucleotides complementary to those preceding the poly(A) tail and flanked at the 5' end with six nucleotides representing a *Xba*I cloning site plus two additional C residues. The reverse transcription reaction was carried out at 42°C for 15 min, followed by enzyme inactivation by heating at 99°C for 5 min and cooling to 5°C for 5 min (one cycle) in a Perkin Elmer Gene Amp PCR System 9600 Thermocycler.

The complementary plus-strand DNA was synthesized by PCR at addition of synthetic oligonucleotide primer B (5' CCGGATCCCAATCCATCATGATGATTC 3'), which is identical in sequence to positions 27 to 50 at the 5' end of the M mRNA, preceded at its 5' end by six nucleotides representing a *Bam*HI cloning site and two additional C residues. The double-stranded DNA was amplified by PCR with *Taq* polymerase, using both oligonucleotides A and B (94°C for 30 min, 55°C for 30 min, and 72°C for 30 min: 25 cycles). The amplified PCR product was purified from a 1% agarose gel and ligated into vector pCR3 according to the manufacturer's instructions (Eukaryotic TA Cloning Kit-Bidirectional, Invitrogen). Two microliters of the ligation reaction was transformed into One Shot TOP10F⁺ competent cells the following day, and 10 colonies were screened for the presence of the M cDNA fragment. One colony containing the mutant M gene in the proper orientation was identified by restriction mapping [pCR1-M (+)] and noncoding [pCR1-M (-)] orientations was determined. The wt M sequence from VSV Indiana, HR-C serotype, kindly provided by S. Beaussart and L. Pelletier (Université de Québec à Montréal), was used for comparison with the sequence of the T1026R1 M gene.

Transient transfection using calcium phosphate and poly(DI-poly(C) induction of the IFN promoter. BHK cells (2×10^6) were passed approximately 24 h prior to transfection into 60-mm-diameter plates. Cells were transfected by the calcium phosphate method (Stratagene Mammalian Transfection kit) according to the manufacturer's directions, with minor modifications. Briefly, the cells were refed approximately 1 to 3 h before transfection with 3 ml of complete medium minus antibiotics. The transfection was carried out with 10 μ g of DNA/60-mm-

diameter plate, 5 μ g of pTWU54, and 5 μ g of the second plasmid. The transfection mix was added to the cells dropwise. After exposure of the cells to the mixture for 5 h, the solution was removed and the cells were subjected to a 1-min glycerol (20% [v/vol]) shock. Following three washes with DME, the cells were refed with 5 ml of complete medium and incubated for approximately 18 to 24 h.

To induce the IFN promoter, cells were treated with 5 ml of complete medium containing 50 μ g of poly(DI-poly(C)), 10 μ g of DEAE-dextran, and 50 μ g of cycloheximide per ml for 6 h. The cells were washed twice with DME, refed with complete medium, incubated for an additional 24 h, and harvested for CAT assay (see below).

Transient transfection using LipofectAMINE and poly(DI-poly(C) induction of the IFN promoter. BHK or COS-1 cells were transiently transfected by using the LipofectAMINE reagent (Gibco-BRL) according to the manufacturer's directions. A total of 2×10^6 to 2.5×10^6 cells per 35-mm-diameter plate were seeded the day before transfection in complete medium. Cells were transfected with the amounts of plasmid DNA indicated in the figure legends. COS cells were transfected with 6 μ l of LipofectAMINE, and BHK cells were transfected with 8 μ l of LipofectAMINE. Cells were exposed to the lipid-DNA complex for 5 h. An equal volume of medium containing twice the amount of serum was added, and the cells were incubated for 18 to 24 h. Fresh complete medium was then added, and the cells were incubated for an additional 18 to 24 h. DNA and LipofectAMINE volumes were scaled up based on surface area when 60-mm-diameter plates were used.

After approximately 18 to 24 h of transfection, COS cells transiently cotransfected with the plasmids were treated with poly(DI-poly(C)) as described above. After transfection for 37 to 41 h, BHK cells transiently transfected with the same plasmids were treated with poly(DI-poly(C)) as described above except that cells were incubated for 4.5 h instead of 6 h before being refed and incubated for 2.5 h. Cells were collected and prepared for CAT analysis. All incubations were done at 37°C.

CAT analysis. CAT activity was determined by the method of Gorman et al. (16), with slight modifications. The cells were disrupted by three freeze-thaw cycles and spun in a microfuge for 15 min at 4°C. One-tenth microliter of [¹⁴C]chloramphenicol (95.50 mCi/mmol; New England Nuclear) was used per sample. The separated acetylated chloramphenicol forms were visualized by autoradiography for approximately 24 h. The results were quantitated with a Packard Instruments InstantImager. The percent acetylation was calculated as follows: (cpm in acetylated forms of [¹⁴C]chloramphenicol/total label in all bands in the lane) $\times 100$.

Immunoprecipitation. Cells were infected with wt VSV at a multiplicity of 20 for 3 to 4 h followed by labeling for 2 h in a mixture containing prewarmed DME minus amino acids, 1X amino acid mix without methionine, and 80 μ Ci of [³⁵S]methionine per ml. Cells for transfection were seeded on 60-mm-diameter wells and transiently transfected by using LipofectAMINE. After 28 to 30 h of transfection, cells were labeled with 1.5 ml of prewarmed DME minus amino acids, 2% fetal bovine serum (COS cells) or 1% calf serum, 1% tryptone phosphate broth (BHK cells), 1X amino acid mix without methionine, and 80 μ Ci of [³⁵S]methionine per ml. Proteins were labeled for 12 to 16 h at 37°C. After labeling, cells were washed twice with ice-cold phosphate-buffered saline, and 900 μ l of radioimmunoprecipitation assay buffer (10 mM Tris-HCl [pH 7.4], 1% Triton X-100, 0.15 M NaCl, 1 mM phenylmethylsulfonyl fluoride) was added to the monolayer. Cells were scraped into a microfuge tube and incubated on ice for 30 min. Cell debris was removed by centrifugation for 3 min. The clarified supernatant was adjusted to 0.2% sodium dodecyl sulfate and incubated with VSV polyclonal antibody 6794 (generously provided by E. Kretzschmar and J. Rose, Yale University) for 30 min at 37°C. Protein A-agarose was added to each sample, and the samples were incubated for 2 h at 37°C while being rotated on a revolving wheel. The pelleted beads were washed three times with immunoprecipitation assay buffer, resuspended in 1X sample buffer, boiled, and applied to a sodium dodecyl sulfate-10% polyacrylamide gel (19). After electrophoresis, the fixed and dried gel was autoradiographed.

RESULTS

Inducibility of the IFN promoter in transiently transfected cells. The goal of this study was to determine whether the wt VSV M protein inhibits transcription from a cellular (IFN) promoter in addition to transcription from the SV40, CMV, and HIV-1 viral promoters studied previously (4, 29). The general experimental strategy was to cotransfect cells with pTWU54, the IFN promoter-CAT reporter gene construct described in previous reports (6, 38), and one of the five VSV genes attached to the SV40 late promoter. These transfected cells were then subjected to poly(DI-poly(C)) treatment to activate the IFN promoter. After a certain time period (see Materials and Methods), cells were lysed and the effect of the cotransfected viral gene on IFN promoter activity was measured by CAT assays.

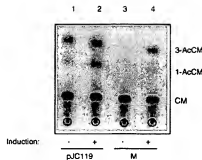


FIG. 1. Dependence of IFN promoter transcription on inducer. BHK cells were cotransfected by the calcium phosphate method with 5 μ g of pTWU54 and 5 μ g of the plasmid mentioned below. After transfection for approximately 24 h, cells in lanes 2 and 4 were induced by poly(I)-poly(C) treatment for approximately 24 h as described in Materials and Methods. Cell lysates were prepared, and their CAT activity was determined by thin-layer chromatography. The cotransfected viral plasmids and the amount of conversion of [14 C]chloramphenicol to acetylated forms are as follows: lanes 1 and 2, pJC119, 3.1 and 15.6%, respectively; and lanes 3 and 4, pKOM 1 (M), 0.5 and 2.2%, respectively. Abbreviations: CM, chloramphenicol; 1-AcCM, 1-acetyl chloramphenicol; 3-AcCM, 3-acetyl chloramphenicol.

IFN induction in virus-infected cells requires synthesis or activation of an IFN inducer. In wt VSV-infected cells, one or more suppressors of IFN induction that effectively prevent expression of the IFN gene are also presumably synthesized (23). By providing an IFN inducer [poly(I)-poly(C)] in the transfection experiments, we measured only the suppressor activity of the product of the viral gene under analysis on the target gene.

BHK cells were selected for these studies because of their high level of transient transfection. In addition, this cell line is a good host for VSV and was used by Black and Lyles (4) in their studies on transcriptional inhibition by VSV M protein. To be able to compare our results to theirs, we used the same cell line. Cells were transfected by using either calcium phosphate or LipofectAMINE. The two reagents gave similar results and were used interchangeably in these studies.

The dependence of the IFN-CAT gene on poly(I)-poly(C) for induction was tested because of the possibility that one of the plasmid preparations contained residual RNA contaminants that could induce the IFN promoter. BHK cells were cotransfected by the calcium phosphate method with plasmid pTWU54 and a second plasmid carrying the VSV M gene. The control sample was cotransfected with pTWU54 and pJC119, the parental plasmid containing the VSV genes (35). As shown in Fig. 1, cells cotransfected with pTWU54 and pJC119 (lane 1) produced only very small amounts of CAT in the absence of poly(I)-poly(C). The addition of poly(I)-poly(C) (lane 2) resulted in a substantial increase in CAT activity. These results indicated that transcription from the IFN gene promoter was dependent on the presence of an inducer under the conditions of our experiments and that if there were RNA contaminants in the plasmid preparations, their contribution to IFN induction was minimal.

Cotransfection of BHK cells with plasmid pTWU54 and the plasmid containing the VSV M gene resulted in the inhibition of CAT synthesis (Fig. 1; compare lanes 2 and 4), indicating that the M protein inhibited transcription from a cellular promoter as well as from the viral promoters previously reported.

Effect of cotransfection of each of the five VSV genes on IFN promoter activity and cell type specificity of the effect. To determine whether cotransfection of the L, N, G, and P genes with pTWU54 in BHK cells influenced CAT synthesis, we

repeated the previous experiment except that LipofectAMINE was used for the transfections instead of calcium phosphate. Figure 2A shows that the plasmid containing the M gene was the most inhibitory in terms of CAT synthesis. Cotransfection with plasmids containing the G, L, N, and P genes had considerably less effect on CAT synthesis. Similar results were obtained when cells cotransfected with pTWU54 and one of the five VSV gene-containing plasmids for 42 h were induced by infection with T1026R1 for 6 h instead of poly(I)-poly(C) (data not shown).

The VSV genes used in these experiments were under regulation of the SV40 late promoter. Although most of these genes were expressed in BHK cells (as shown in Fig. 3), we also tested their effect on the IFN gene promoter in COS-1 cells, which express the SV40 large T antigen. As in BHK cells, IFN induction was dependent on the addition of poly(I)-poly(C) (Fig. 2B, lanes 1 and 2). Cotransfection of these cells with pTWU54 and the M gene resulted in a substantial decrease in CAT activity (Fig. 2B, lane 5). Cotransfection of these cells with pTWU54 and the G, L, N, and P genes had no significant inhibitory effect on CAT activity (Fig. 2B, lanes 3, 4, 6, and 7).

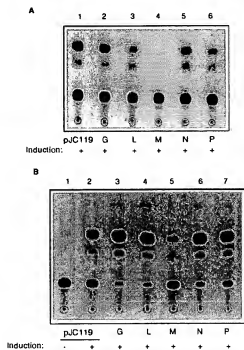


FIG. 2. Effect of the VSV proteins on IFN promoter activity in LipofectAMINE-transfected BHK and COS-1 cells. (A) BHK cells were cotransfected with 1 μ g of pTWU54 and 2 μ g of the viral expression vector indicated below, using the LipofectAMINE reagent. After transfection for approximately 40 h, cells were induced with poly(I)-poly(C) as indicated in Materials and Methods and cell lysates were prepared. CAT activity of cell extracts was determined by thin-layer chromatography. The cotransfected viral plasmids and the amounts of chloramphenicol acetylation are as follows: lane 1, pJC119, 23.5%; lane 2, pSVGL (G), 21.3%; lane 3, pSV-VSL1 (L), 10.6%; lane 4, pKOM 1 (M), 1.8%; lane 5, pJS223 (N), 17.7%; and lane 6, pLH7 (P), 9.8%. (B) COS-1 cells were cotransfected with 0.75 μ g of pTWU54 and 2.25 μ g of viral plasmid for 44 h. Cells were induced with poly(I)-poly(C) as indicated in Materials and Methods, except for one sample that was not induced (lane 1). The cotransfected viral plasmids and the amounts of chloramphenicol acetylation are as follows: lane 1, pJC119, 3.0%; lane 2, pJC119, 40.7%; lane 3, pSVGL (G), 93.8%; lane 4, pSV-VSL1 (L), 94.4%; lane 5, pKOM 1 (M), 18.1%; lane 6, pJS223 (N), 90.8%; and lane 7, pLH7 (P), 78.7%.

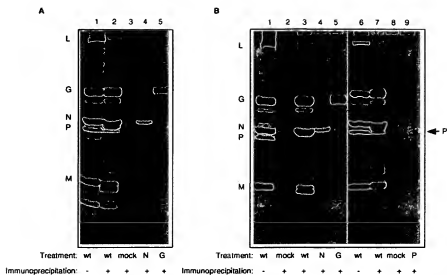


FIG. 3. Immunoprecipitation of VSV proteins in transiently transfected BHK and COS-1 cells. Cells were transfected with 1.25 μ g of pTWU54 and 5 μ g of a second plasmid (indicated below) by the LipofectAMINE method. Following incubation for 33 to 36 h, the cells were incubated for 12 to 15 h in medium containing 80 μ Ci of [35 S]methionine per ml. Lysates were immunoprecipitated with polyclonal VSV antibody (see Materials and Methods). The method for infecting and labeling cells with wt VSV was described in Materials and Methods. (A) BHK cells. Lane 1, infected cell lysate not immunoprecipitated; lane 2, wt VSV-infected cells immunoprecipitated; lane 3, pJC119 immunoprecipitated; lane 4, pJS223 (N) immunoprecipitated; lane 5, pGL (G) immunoprecipitated. (B) COS-1 cells. Lanes 1 and 6, wt VSV-infected cell lysate not immunoprecipitated; lanes 2 and 8, pJC119 immunoprecipitated; lanes 3 and 7, wt VSV-infected cells, immunoprecipitated; lane 4, pJS223 (N) immunoprecipitated; lane 5, pSVGL (G) immunoprecipitated; lane 9, pLH7 (P) immunoprecipitated.

These results demonstrated that in these two different cell lines, the M protein was the most inhibitory VSV protein.

Immunoprecipitation of cells transiently transfected with VSV genes. Immunoprecipitation was used to determine whether the transfected BHK and COS cells were expressing the intended viral gene product. Cells were transfected for a total of 48 h, and the proteins were labeled with [35 S]methionine during the last 12 h of transfection as described in Materials and Methods. After the transfections, cell lysates were prepared and the incorporation of radioactive methionine into virus-specific protein was determined by using immunoprecipitation with a polyclonal antibody to total VSV proteins.

In some cases, cells were infected with wt VSV and labeled with [35 S]methionine. Lysates from these cells served as markers for viral proteins and also showed which VSV proteins were the most antigenic. Figure 3A, lane 2, shows that the polyclonal antibody precipitated proteins G, N, and M best, followed by proteins P and L. When BHK cells were transfected with pTWU54 and the plasmid containing the N or G gene followed by immunoprecipitation with the polyclonal antibody, both the N and G genes appeared to be expressed, since both G and N proteins were immunoprecipitated (Fig. 3A, lanes 4 and 5). No L nor P protein was detected in similar experiments (data not shown), probably because the polyclonal antibody used did not immunoprecipitate these proteins very effectively.

Similar results were obtained when COS cells were used in the double transient transfections instead of BHK cells. The N and G genes were obviously expressed (Fig. 3B, lanes 4 and 5), and a small amount of P protein (Fig. 3B, lane 9) was also detected in these cells. It is not known whether the L gene was expressed in these cotransfection experiments, since the L protein could not be detected. Therefore, the question of whether the L protein affects IFN transcription is still open. However, the L clone used in these experiments has been shown to complement and rescue L gene temperature-sensitive mutants of VSV at the nonpermissive temperature (31). No M gene

expression was detected in either BHK or COS cells by using immunoprecipitation methods, confirming similar observations reported by other laboratories (3, 4, 29).

Effect of each of the VSV proteins on viral promoters. In their studies, Black and Lyles (4) examined the role of M gene expression on CAT reporter gene expression, using the SV40 early (pSV2CAT) promoter. We extended these studies by determining the effect of cotransfection with viral genes L, N, P, and G on the CAT target gene under control of the CMV (pCEP4/CAT) and SV40 promoters. As shown in Fig. 4, lanes 1 to 3, 5, and 6, cotransfection of the CAT reporter gene controlled by the CMV promoter with pJC119 or viral gene G, L, N, or P did not inhibit CAT production. Cotransfection with

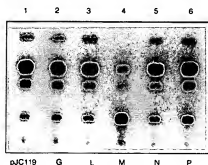


FIG. 4. Effect of the VSV proteins on CAT gene expression controlled by the CMV promoter. BHK cells were cotransfected with 8 ng of pCEP4/CAT and 3 μ g of the viral expression vector indicated below, using the LipofectAMINE reagent. After transfection for approximately 40 h, cell lysates were prepared and their CAT activity was determined as described in Materials and Methods. The cotransfected viral plasmids and the amount of conversion of chloramphenicol to acetylated chloramphenicol were as follows: lane 1, pJC119, 90.8%; lane 2, pSVGL (G), 89.6%; lane 3, pSV-VSL1 (L), 88.9%; lane 4, pJC119 (M), 14.2%; lane 5, pJS223 (N), 85.5%; and lane 6, pLH7 (P), 83.3%.

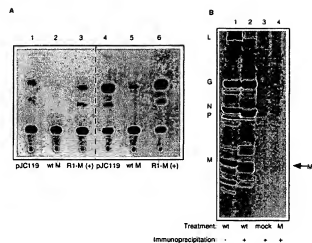


FIG. 5. Effect of the M protein from T1026R1 on the IFN and SV40 early promoters. (A) In lanes 1 to 3, BHK cells were transiently cotransfected with 0.4 μ g of pTWU54 and 1.6 μ g of a viral plasmid, using LipofectAMINE. After approximately 40 h of transfection, cells were induced by poly(I)-poly(C) treatment as described in Materials and Methods. Cells were collected, lysed, and analyzed by CAT assay. In lanes 4 to 6, BHK cells were transiently cotransfected with 25 ng of pSV2CAT and 3 μ g of the indicated viral plasmid. The cotransfected viral plasmids and the amounts of conversion of chloramphenicol to acetylated forms are as follows: lanes 1 and 4, pJC119, 10.1 and 68.0%, respectively; lanes 2 and 5, pKOM 1 (wt M), 1.0 and 3.2%, respectively; and lanes 3 and 6, pCR1-M (+) (R1 M), 12.8 and 70.4%, respectively. (B) Immunoprecipitation of T1026R1 M protein in transfected BHK cells. Cells were cotransfected with 1.25 μ g of pTWU54 and 5 μ g of pCR1-M (+) for 30 h, using the LipofectAMINE method. Cells were then labeled with 80 μ Ci of [35 S]methionine per ml for 12 h following immunoprecipitation with VSV polyclonal antibody. Lane 1, wt virus-infected cells not immunoprecipitated; lane 2, wt virus-infected cells immunoprecipitated; lane 3, cells transfected with pJC119 and immunoprecipitated; lane 4, cells transfected with pCR1-M (+) and immunoprecipitated.

the M gene, on the other hand, caused a large reduction in CAT activity (Fig. 4, lane 4). The same results were obtained when the target CAT gene was controlled by the SV40 early promoter instead of the CMV promoter (data not shown). These results demonstrated that the five VSV proteins affected viral and cellular promoters similarly.

The M protein inhibited CAT activity in a gene dosage-dependent manner (data not shown), as previously demonstrated (4).

Cloning and expression of the T1026R1 M gene. If the VSV M protein were responsible for the inhibition of IFN expression in wt virus-infected cells and our cotransfection experiments were an accurate reflection of the events occurring in infected cells, then cotransfection of pTWU54 and the M gene from the IFN-inducing, host transcription inhibition-negative mutant T1026R1 should not suppress CAT activity. The effect of cotransfecting BHK cells with plasmids containing the wt VSV M gene or the mutant M gene, cloned into vector pCR3 containing a CMV promoter, on CAT expression from either the IFN or the SV40 early promoter was examined. The results (Fig. 5A) demonstrated that expression of the M gene from the mutant did not result in the inhibition of CAT transcription from either the IFN (lanes 1 to 3) or the SV40 (lanes 4 to 6) promoter. The same results were obtained with plasmid pCR3 containing the T1026R1 M gene in the noncoding orientation (data not shown). These results indicated that the mutant M protein was defective in its ability to suppress expression from both a cellular and a viral promoter.

Expression of the T1026R1 M gene during cotransfection was verified by immunoprecipitation (Fig. 5B) and RT-PCR

(data not shown). T1026R1 M protein was detected but at a rather low level. While this mutant protein was defective in transcription inhibition, it was like the wt protein in causing cytopathic cell rounding (14). The low amount of M protein detected may have resulted from this function of the protein.

The nucleotide sequence of the cloned T1026R1 M gene. The nucleotide sequence of the cloned mutant M gene was determined as described in Materials and Methods and aligned to that of the M gene from the wt Indiana HR strain of VSV. Two nucleotide changes were detected. Each led to amino acid changes. One was a methionine-to-arginine change in amino acid 51, and the other was an aspartic acid-to-glycine change in amino acid 92. Beausoleil and Poliquin (1) sequenced two independent T1026R revertants and found only the one change in amino acid 51. As discussed later, the mutation in position 51 is the same as that found by Coulon et al. (12) in mutant α 802, which is defective in host transcription inhibition. Whether the mutation in amino acid 92 is an independent mutation of T1026R1 arising in our laboratory after many passages of the original stock virus or an artifact produced during the PCR step has yet to be determined.

DISCUSSION

In this study, three points were considered: the effect of the wt VSV M protein on transcription of a target gene from a cellular (IFN) promoter, the cloning and sequencing of the M gene from the IFN-inducing VSV mutant T1026R1, and the effect of this mutant M protein on transcription from both cellular and viral promoters.

Lack of promoter specificity. The M protein has been shown to be a very powerful inhibitor of transcription of target genes controlled by various viral promoters (4, 29). These studies demonstrated that expression of the CAT reporter gene from a cellular promoter was as susceptible to this inhibitor of transcription as expression from viral promoters. This finding suggests that the M protein acts on the general transcription apparatus and does not possess specificity in the regulation of viral and cellular (at least the IFN) promoters. This result was of particular interest because the IFN promoter could have been regulated differently from viral promoters as a result of the major role that IFN plays in host cell defense against viral infection.

This finding, however, does not preclude the possibility that VSV affects host transcription differentially from specific promoters by other mechanisms. Indeed, a nuclear protein that binds specifically to IFN-stimulated response elements (ISRE) is activated shortly after L929 cells are infected with VSV or are treated with poly(I)-poly(C) (7). Activation in the former case requires VSV primary transcription and tyrosine kinase activity (7). What is interesting about this finding is that while transcription of a reporter gene not containing ISRE in its promoter was severely inhibited in VSV-infected cells as a result of host transcription shutoff (presumably by the M protein), transcription of the same reporter gene attached to ISRE was much less reduced (7). Presumably, the VSV-activated ISRE-binding protein was responsible for the differential sensitivity of these two genes to transcription inhibition.

The viral G, N, and P proteins did not possess the potent transcriptional inhibitory properties of the M protein, as measured by CAT assays (Fig. 2 and 4). This was true even when equal molar concentrations of the viral DNAs were used in the transfections (data not shown).

The human IFN- β promoter used in these experiments contained positions -286 to +67 of the regulatory DNA region (38). This region encompasses all known major promoter ele-

ments, including the high-mobility-group binding sites (13). Positions -125 to -38 of the IFN- β promoter region (relative to the cap site) are considered to comprise the minimal sequence required for full IFN induction by virus infection or poly(I)-poly(C) treatment (15).

Cloning of the T1026R1 M gene. The cloning and sequencing of the T1026R1 M gene provided interesting results as to which amino acid was responsible for the lack of inhibition of transcription in cells infected by this virus. Comparison of the sequence of the cloned T1026R1 M gene with that of its wt parent indicated two amino acid changes. Interestingly, the methionine-to-arginine change in amino acid 51 was the same as that found in mutant ts082, which is defective in transcription inhibition in chicken embryo fibroblasts at the nonpermissive temperature (12). This mutant is also an excellent inducer of IFN at the nonpermissive temperature in chicken embryo fibroblasts and other cell lines (24). The importance of amino acid 51 in transcription was demonstrated by the isolation of temperature-resistant revertants of ts082 in which the wt phenotype in terms of host transcription inhibition and IFN induction was reestablished (12, 24). The M gene from the revertant had the same sequence as the original wt M gene from which the ts082 mutant was derived (12).

Black et al. (3) reconstructed the methionine-to-arginine change in the M gene from mutant ts082 in an experimental M gene and found that in cotransfections with the CAT reporter gene, it had no effect on transcription of the reporter gene but behaved like the wt gene in its ability to function in virus assembly. Together, these data suggest that amino acid 51 in the M protein plays an important role in the ability of the M protein to inhibit host transcription.

Lack of inhibition of transcription by the cloned T1026R1 M gene. The M protein from mutant T1026R1 did not inhibit expression of CAT from either the IFN promoter or the SV40 promoter (Fig. 5). This finding is consistent with the finding that this mutant does not inhibit host cell transcription until relatively late in the infection. Although expression of the T1026R1 M gene was detected by both RT-PCR and immunoprecipitation, it was not as abundantly expressed as some of the other VSV genes, which was rather surprising since it had little effect on host transcription. We attributed this to the cytopathic cell rounding function of the M protein, which is like that of the wt protein in this mutant.

Relationship of the inhibition of IFN gene expression by M protein to the regulation of IFN induction in wt VSV-infected cells. The results reported in this study demonstrated that the M protein inhibited gene expression from the IFN promoter in BHK and COS-1 cells. The question that we pose is whether the M protein regulates IFN gene expression in wt virus-infected cells. Virus induction of the IFN- β promoter is a multistep process requiring the synergistic interactions between distinct virus-inducible elements and several different transcription factors, including HMG1(Y), NF- κ B, and ATF-2 (13). In theory, any of the steps leading to activation of this promoter could be modified or inactivated by virus infection even before the full transcription complex is assembled. The experimental approach used in this study bypassed an essential step in the virus-directed activation of the IFN gene. The IFN gene promoter was induced with poly(I)-poly(C) or by infection with VSV mutant T1026R1. While IFN is induced under these conditions, the control of IFN induction in cells infected by the wt virus is more complex.

In wt VSV-infected cells, viral gene products may interact with cellular factors to provide levels of IFN promoter regulation that would not have been evident in the experiments in this report. For example, activation of transcription factor NF-

κ B, which entails its translocation to the nucleus (reviewed in reference 17), is one of the first steps in IFN gene induction (13). In mouse L cells infected with VSV mutant T1026R1, NF- κ B is activated within the first 30 min to 1 h after virus adsorption (6). In contrast, there is a delay of up to at least 4 h in cells similarly infected with wt VSV. By the time NF- κ B is activated in this latter case, host transcription is inhibited to an extent that any gene transcription, including IFN gene transcription, is precluded. Some evidence exists that cells infected with wt VSV may contain an inhibitor of NF- κ B activation. Preliminary data suggest that the inhibitor may be different from the general transcriptional inhibitor (M protein) discussed in this report, based on UV target size analysis (6). The nature and role of this NF- κ B activation inhibitor is not known at this time, but it may contribute to the overall regulation of IFN induction. These results suggest that the IFN gene may be regulated at both induction and transcription levels. Mutant T1026R1 may have defective genes that act at both of these levels.

A great deal of our knowledge about the regulation of IFN by VSV derives from studies done in aged primary chicken embryo cells. Because there are significant differences in how VSV affects the process of IFN induction in avian and mammalian cells (26, 37), it is particularly difficult to compare results obtained in these two cell types. Indeed, studies by Marcus et al. (24) suggest that the M protein is not involved in the regulation of IFN gene expression in primary chicken embryo cells. They compared the abilities of different field isolates of VSV to induce IFN in these avian cells and found that one isolate (22-20) was an excellent inducer of IFN whereas another isolate (22-25) did not induce IFN, yet the M gene sequence was identical in the two isolates. In mouse L cells, isolate 22-25 also did not induce IFN, but in contrast to avian cells, isolate 22-20 induced only a very small amount of IFN (33).

Since chicken embryo fibroblasts are sensitive to the effects of M protein on host transcription (12), the fact that IFN induction occurs in cells infected with isolate 22-20, which has a wt M gene, is a paradox. These data could be explained if, indeed, two VSV genes, including the M protein gene, are involved in the control of IFN gene transcription. While VSV mutant T1026R1 may be defective in both genes, as argued above, isolate 22-20 may be defective in only one, the one responsible for limiting IFN induction at an early step of transcription. Why the M protein does not prevent transcription of the IFN gene after this step in chicken embryo fibroblasts could be explained if the rate of host transcription inhibition were slow in comparison to the rate of transcription initiation complex formation (induction). In mouse L cells, host transcription inhibition is so rapid that even if the early steps of transcription were to take place, transcription of the IFN gene would be precluded. As mentioned above, very little IFN was induced by isolate 22-20 in these cells (33).

In sum, this report represents the first demonstration that the M protein from wt VSV indiscriminately inhibits reporter gene transcription from cellular and viral promoters and that a mutant M protein from VSV T1026R1 with a methionine-to-arginine change in position 51 of the protein is no longer active in this capacity. While the results presented are consistent with a role of M protein in IFN gene regulation in VSV-infected mammalian cells, specific regulation of the IFN gene promoter probably occurs at another step in transcription.

ACKNOWLEDGMENTS

We thank P. I. Marcus and M. J. Sckellack for valuable advice and input in these studies, Carol Menditto for technical assistance, Dawna

Houston for quantitating the CAT assays, R. Schloemer for providing the pTWU54 plasmid, M. Schubert for providing the VSV expression vectors, J. Rose and E. Kretzschmar for the VSV polyclonal antibody, and S. Beauvolet and L. Poliquin for the sequence of the M gene from the HR strain of VSV (Indiana).

This research was supported by the University of Connecticut Research Foundation and by USDA grant 93-37204-9335.

REFERENCES

- Beauvolet, S., and L. Poliquin. Personal communication.
- Beckes, J. D., L. C. Childers, and J. Perrault. 1989. Phosphorylation of vesicular stomatitis virus M protein: evidence for a second virion-associated protein serine kinase activity. *Virology* 169:161-171.
- Black, B. L., R. B. Rhodes, M. McKenzie, and D. S. Lyles. 1993. The role of vesicular stomatitis virus matrix protein in inhibition of host-directed gene expression is genetically separable from its function in virus assembly. *J. Virol.* 67:4814-4821.
- Black, B. L., and D. S. Lyles. 1992. Vesicular stomatitis virus matrix protein inhibits host cell-directed transcription of target genes in vivo. *J. Virol.* 66:4058-4064.
- Blondel, D., G. S. Harrison, and M. Schubert. 1990. Role of matrix protein in cytopathogenesis of vesicular stomatitis virus. *J. Virol.* 64:1716-1725.
- Boulares, A. H., M. C. Ferran, and J. Lucas-Lenard. 1996. NF- κ B activation is delayed in mouse L929 cells infected with interferon suppressing, but not inducing, vesicular stomatitis virus strains. *Virology* 218:71-80.
- Bovolenia, C., J. Lou, Y. Kanno, B.-K. Park, A. M. Thornton, J. E. Coligan, M. Schubert, and K. Ozato. 1995. Vesicular stomatitis virus infection induces a nuclear DNA-binding factor specific for the interferon-stimulated response element. *J. Virol.* 69:4173-4181.
- Carroll, A. R., and R. R. Wagner. 1979. Role of the membrane (M) protein in endogenous inhibition of in vitro transcription of vesicular stomatitis virus. *J. Virol.* 29:134-142.
- Chong, L. D., and J. K. Rose. 1994. Interaction of normal and mutant vesicular stomatitis matrix proteins with the plasma membrane and nucleocapsid. *J. Virol.* 68:441-447.
- Chong, L. D., and J. K. Rose. 1993. Membrane association of functional vesicular stomatitis virus matrix protein in vivo. *J. Virol.* 67:407-414.
- Clinion, G. M., S. P. Little, F. S. Hagen, and A. S. Huang. 1978. The matrix (M) protein of vesicular stomatitis virus regulates transcription. *Cell* 15:1455-1462.
- Coulson, P., Y. Deutsch, F. Lafay, C. Martinet-Edelstein, F. Wyers, R. C. Herman, and A. Flamand. 1990. Genetic evidence for multiple functions of the matrix protein of vesicular stomatitis virus. *J. Gen. Virol.* 71:991-996.
- Du, W., D. Thanos, and T. Maniatis. 1993. Mechanisms of transcriptional synergism between distinct virus-inducible enhancer elements. *Cell* 74:887-898.
- Ferran, M. C., and J. Lucas-Lenard. Unpublished data.
- Fujita, T., S. Ohno, H. Yasunuma, and T. Taniguchi. 1985. Delimitation and properties of DNA sequences required for the regulated expression of human interferon- β genes. *Cell* 41:489-496.
- Gorman, C. M., L. F. Moffatt, and B. H. Howard. 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* 2:1044-1051.
- Grimm, S., and P. A. Baeuerle. 1993. The inducible transcription factor NF- κ B: structure-function relationship of its protein subunits. *Biochem. J.* 290:297-308.
- Hudson, L. D., C. Corda, and R. A. Lazzarini. 1986. Cloning and expression of a viral phosphoprotein: structure suggests vesicular stomatitis virus NS may function by mimicking an RNA template. *J. Gen. Virol.* 67:1511-1519.
- Lammeli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.
- Lyles, D. S., L. Puddington, and B. J. McCreedy, Jr. 1988. Vesicular stomatitis virus M protein in the nuclei of infected cells. *J. Virol.* 62:4387-4392.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Marcus, P. I., M. J. Skellick, and S. T. Nichol. 1992. Interferon induction by viruses. XXI. Vesicular stomatitis virus: interferon inducibility as a phylogenetic marker. *J. Interferon Res.* 14:71-79.
- Marcus, P. I., and M. J. Skellick. 1987. Interferon induction by viruses. XV. Biological characteristics of interferon induction-suppressing particles of vesicular stomatitis virus. *J. Interferon Res.* 7:269-284.
- Marcus, P. I., M. J. Skellick, C. F. Spiranopoulos, and S. T. Nichol. 1993. Interferon induction by viruses. XXII. Vesicular stomatitis virus-Indiana: M-protein and leader RNA do not regulate interferon induction in chicken embryo cells. *J. Interferon Res.* 13:413-418.
- Marcus, P. I., and M. J. Skellick. 1994. Interferon induction: regulation by both virus and cell. *Hokkaido J. Med. Sci.* 69:1320-1331.
- Marcus, P. I. 1987. Personal travels and travails with the interferon system. *J. Interferon Res.* 7:471-479.
- McCreedy, B. J., and D. S. Lyles. 1989. Distribution of M protein and nucleocapsid protein of vesicular stomatitis virus in infected cell plasma membranes. *Virus Res.* 14:189-206.
- Melki, R., Y. Gaudin, and D. Blondel. 1994. Interaction between tubulin and the viral matrix protein of vesicular stomatitis virus: possible implications in the viral cytopathic effect. *Virology* 202:339-347.
- Paik, S.-Y., A. C. Banerjee, G. G. Harrison, C.-J. Chen, and M. Schubert. 1995. Inducible and conditional inhibition of human immunodeficiency virus proviral expression by vesicular stomatitis matrix protein. *J. Virol.* 69:3529-3537.
- Rose, J. K., and J. E. Bergmann. 1982. Expression from cloned cDNA of cell-surface secreted forms of the glycoprotein of vesicular stomatitis virus in eucaryotic cells. *Cell* 30:753-762.
- Schubert, M., G. G. Harrison, C. D. Richardson, and E. Meier. 1985. Expression of a cDNA encoding a functional 241-kilodalton vesicular stomatitis virus RNA polymerase. *Proc. Natl. Acad. Sci. USA* 82:7984-7988.
- Skellick, M. J., and P. I. Marcus. 1979. Persistent infection. II. Interferon-inducing temperature sensitive mutants as mediators of cell sparing: possible role in persistent infection by vesicular stomatitis virus. *Virology* 95:36-47.
- Skellick, M. J. Personal communication.
- Simon, K. O., P. A. Whitaker-Dowling, J. S. Youngner, and C. C. Wundell. 1990. Sequential disassembly of the cytoskeleton in BHK21 cells infected with vesicular stomatitis virus. *Virology* 177:289-297.
- Sprague, J., J. H. Condra, H. Arnheiter, and R. A. Lazzarini. 1983. Expression of a recombinant DNA gene coding for the vesicular stomatitis virus nucleocapsid protein. *J. Virol.* 45:773-781.
- Stanners, C. M., A. M. Francouer, and T. Lam. 1977. Analysis of VSV mutant with attenuated cytopathogenicity: mutation in viral function, P, for inhibition of protein synthesis. *Cell* 11:273-281.
- Svitlik, C., and P. I. Marcus. 1985. Interferon induction by viruses. XII. Inhibition of protein synthesis renders early chick embryo cells refractory to interferon induction. *J. Gen. Virol.* 66:883-886.
- Twu, J.-S., and R. H. Schloemer. 1987. Transcriptional *trans*-activating function of hepatitis B virus. *J. Virol.* 61:3448-3453.
- Wagner, R. R., and A. S. Huang. 1966. Inhibition of RNA and interferon synthesis in krebs-2 cells infected with vesicular stomatitis virus. *Virology* 28:1-10.
- Wagner, R. R. 1987. Rhabdovirus biology and infection: an overview, p. 9-74. In R. Wagner (ed.), *The rhabdoviruses*. Plenum Publishing Corp., New York, N.Y.
- Wertz, G. W., and J. S. Youngner. 1970. Interferon production and inhibition of host cell synthesis in cells infected with vesicular stomatitis virus. *J. Virol.* 6:476-484.
- Ye, Z., W. Sun, K. Saryanarayana, P. Justice, D. Robinson, and R. R. Wagner. 1994. Membrane-binding domains and cytopathogenesis of the matrix protein of vesicular stomatitis virus. *J. Virol.* 68:7386-7396.

Guide for Authors

AIMS AND SCOPE

Virology publishes the results of original basic research on viruses of animals (vertebrate and invertebrate), plants, bacteria, and yeasts/fungi. We invite articles on all areas of research, including virus replication and gene expression, virus structure and assembly (including atomic structure), virus-cell interaction (including cellular changes as a consequence of viral infection), viral pathogenesis and immunity (at both molecular and organismal levels), viral vectors/gene therapy, and molecular aspects of prevention of viral infection. Papers describing results on emerging viruses and unconventional agents will receive special attention.

EDITORS

The editors and their areas of responsibility are [given here](#)

CATEGORIES AND TYPES OF ARTICLES

Papers will be published in *Virology* under one of the following subheadings:

- Virus Replication/Gene Expression
- Virus Structure and Assembly
- Virus-Cell Biology
- Viral Pathogenesis and Immunity
- Gene Therapy/Viral Vectors
- Emerging Viruses/Unconventional Agents

Virology publishes three forms of manuscripts: (a) regular manuscripts, including short but complete studies; (b) rapid communications; and (c) minireviews.

Regular manuscripts present the results of original basic research in all areas of virology (above) that break new ground and serve as a valuable addition to the literature in the field.

A Rapid Communication is a brief, definitive report of highly significant and timely findings in the field. Authors should indicate the submission as such, and if on preliminary inspection the editor believes the paper is of a nature to warrant this category, the paper will receive very rapid review and, if acceptable, will be published within an average of 8 weeks from receipt.

Minireviews bring cutting-edge developments and themes in the field to virologists, postdoctoral fellows, graduate students, and others interested in the field. The goal of these minireviews is to focus on a sharply defined topic in an interesting area in virology or on recent research (such as two or three papers coming in a specific area of virology). The objective is to make the information accessible to researchers who work in other areas of virology. Minireviews should be pithy, that is, should not cover the field in question comprehensively but rather address fundamental concepts, challenges, and problems in the field. In summary, virologists and others, both directly in and outside the area of the minireview, should benefit from reading these minireviews. The minireviews should provide a critical view of the field. Minireviews would also be an appropriate forum for introducing new viewpoints, indicating important issues to be addressed, and challenging concepts.

SPEED OF PUBLICATION

Peer Review Process: Each editor is responsible for having manuscripts reviewed and for making the final decision concerning the disposition. Each manuscript is reviewed by at least two reviewers. All manuscripts are reviewed as rapidly as possible, and an editorial decision is usually reached within 4 to 5 weeks of the manuscript's submission.

Publication Schedule: Accepted manuscripts are usually published in print within 12-14 weeks of acceptance, and within 4-5 weeks from receipt of authors corrected proofs. Please note that early publication online may occur within days of receipt of corrected proofs.

EDITORIAL POLICIES

There are no page charges for publication in *Virology*.

Authors should understand that space limitations make it impossible to publish manuscripts that describe work that does not break new ground conceptually, particularly if the work merely mirrors work done on a closely related virus without adding significant new knowledge, such as partial sequences of related viruses, evolutionary studies based on partial sequences, or descriptions of antigenic relationships and epitopes. Exceptions will be made, however, if these data reveal new insights into the fundamental properties of the virus.

Submission of an article implies that the work described has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, without the written consent of the Publisher.

Publication of a research article in *Virology* is taken to imply that the authors are prepared to distribute freely to academic researchers for their own use any materials (e.g., viruses, cells, DNA clones, antibodies) used in the published experiments. Prior to publication, nucleotide sequences and protein sequences must be deposited with GenBank (Web site: <http://www.ncbi.nlm.nih.gov/Genbank/>) and an accession number obtained for publication in the manuscript. X-ray crystallographic coordinates must be deposited with the Protein Data Bank (Web site: <http://www.rcsb.org/pdb/>).

Upon acceptance of an article, authors will be asked to transfer copyright (for more information on copyright, see <http://authors.elsevier.com>). This transfer will ensure the widest possible dissemination of information. A letter will be sent to the corresponding author confirming receipt of the manuscript. A form facilitating transfer of copyright will be provided after acceptance.

If material from other copyrighted works is included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article. Elsevier has preprinted forms for use by authors in these cases: contact Elsevier Global Rights Department, P.O. Box 800, Oxford OX5 1DX, UK; phone: (+44) 1865 843830, fax: (+44) 1865 853333, e-mail: permissions@elsevier.com. Requests may also be completed on-line via the Elsevier home page (<http://www.elsevier.com/locate/permissions>).

NEW!

SUBMISSION OF MANUSCRIPTS

Authors are requested to submit their papers electronically by using online manuscript submission available at <http://ees.elsevier.com/viro>. This site will guide authors stepwise through the submission process. Authors can upload their articles as Microsoft (MS) Word, WordPerfect, or LaTeX files. It is also possible to submit an article in PostScript or Adobe Acrobat PDF format, but if the article is accepted, the original source files will be needed. If you submit a word processing file, the system generates an Adobe Acrobat PDF version of the article, which is used for the reviewing process.

The above represents a very brief outline of this form of submission. It can be advantageous to print this Guide for Authors section from the site for reference in the subsequent stages of article preparation.

Submission Checklist: You will be asked to submit:

- **Cover Letter:** Document (Word, WordPerfect, RTF, PDF, LaTeX) containing your cover letter to the Editors.
- **Response to Reviews** (Resubmissions Only): Document (Word, WordPerfect, RTF, PDF, LaTeX) detailing your response to the reviewers' and editor's comments of a previously rejected manuscript that you are resubmitting.
- **Manuscript:** Single word processing (Word, WordPerfect, RTF) or LaTeX file consisting of the title page, abstract, manuscript text, and any figure/table legends.
- **Tables:** Tables should be separate from the manuscript text and can be uploaded individually or consolidated into a single file. The file description you input when uploading your table must include the table number or range (e.g., Table 1, Tables 2-4).
- **Figures:** Figures should be uploaded individually as TIF or EPS files. Although other figure formats are allowed by the system (GIF, JPEG, Postscript, PICT, PDF, Excel, and PowerPoint), they will delay the production process should your manuscript be accepted. The file description you input below when uploading your figure must include the figure number (e.g., Fig. 2A).

Please submit, with the manuscript, the names and addresses of up to 6 potential referees.

FORMATS AND MANUSCRIPT STYLE

It is important that the file be saved in the native format of the word processor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced during typesetting. In particular, do not use the word processor's options to justify text or to hyphenate words. However, do use boldface, italics, subscripts, superscripts, etc. Do not embed graphically designed equations or tables, but prepare these using the word processor's facility. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. To avoid unnecessary errors you are strongly advised to use the spell checking function of your word processor. See also the section on preparation of electronic illustrations.

Authors in Japan kindly note that, upon request, Elsevier Japan will provide a list of people who can check and improve the English of an article (before submission). Please contact our Tokyo office: Elsevier, 4F Higashi-Azabu, 1 Chome Bldg., 1-9-15 Higashi-Azabu, Minato-ku, Tokyo 106-0044, Japan; tel: +81-3-5561-5032; fax: +81-3-5561-5045; e-mail: jp.info@elsevier.com.

ORGANIZATION AND GENERAL STYLE OF MANUSCRIPTS

Manuscripts should be written as concisely as possible with minimum repetition between Results and Discussion and Materials and Methods and figure legends. All figures must be of a quality such that the reviewers can judge the data.

(a) Regular Manuscripts

The organization shown below should be followed (in the order given):

The **title page** should contain the following elements:

The *title* should be concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulas where possible.

Author names and affiliations. Where the family name may be ambiguous (e.g., a double name), please indicate this clearly. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lowercase superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name, and, if available, the e-mail address of each author.

Corresponding author. Clearly indicate who is willing to handle correspondence at all stages of refereeing and publication, also postpublication. Ensure that telephone and fax numbers (with country and area code) are provided in addition to the e-mail address and the complete postal address.

Present/permanent address. If an author has moved since the work described in the article was done, or was visiting at the time, a present address (or permanent address) may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract. A concise and factual abstract is required (maximum length 150 words). The abstract should summarize the main findings of the paper. An abstract is often presented separate from the article, so it must be able to stand alone. References should therefore be avoided, but if essential, they must be cited in full, without reference to the reference list.

Keywords. Immediately after the abstract, provide a maximum of 10 keywords, avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing and searching purposes.

The **Introduction** should be succinct with no subheadings. It should contain material directly relevant to the research that is described and should state clearly the aims of the investigation in the light of related work. Fair citation of the work of others is essential. Authors are asked to use nomenclature approved by the International Committee for the Taxonomy of Viruses (ICTV) (Web site: <http://www.ncbi.nlm.nih.gov/>) the first time a virus name appears. Commonly used vernacular names may be used after viruses are first correctly identified. Genetic loci should be italicized; protein products of the loci are not italicized.

Results and Discussion may be divided by subheadings or may be combined into one section when substantial redundancy cannot be avoided in two separate sections or if a long discussion is not warranted. A Discussion section should be constructively interpretive and not restate experimental data.

Materials and methods should provide sufficient information to permit the work to be repeated and should be kept concise by referring to previously published procedures. With increasing studies on pathogenicity of viruses, it is important that the provenance of viruses be stated clearly.

Acknowledgments. Place acknowledgments, including information on grants received, before the references, in a separate section, and not as a footnote on the title page.

References should include only articles that have been published or are in press. Unpublished data, submitted manuscripts, or personal communications should be cited within the text. Personal communications should be documented by a letter of permission. Abstracts of work presented at meetings may not be cited. Names of authors should be mentioned in the text with year of publication in parentheses. References should be listed alphabetically at the end of the paper. Journal names should be abbreviated according to the Chemical Abstracts Service index: <http://www.cas.org/>.

Text. All citations in the text should refer to:

1. Single author: the author's name (without initials, unless there is ambiguity) and the year of publication.
2. Two authors: both authors' names and the year of publication.
3. Three or more authors: first author's name followed by "et al." and the year of publication.

Citations may be made directly (or parenthetically). Groups of references should be listed first alphabetically, then chronologically.

Examples: "as demonstrated (Allan, 1996a, 1996b, 1999; Allan and Jones, 1995). Kramer et al. (2000) have recently shown"

List. References should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters "a", "b", "c", etc., placed after the year of publication.

Reference to a journal publication:

Park, J., Nadeau, P.E., Mergia, A., 2002. A minimal genome simian foamy virus type 1 vector system with efficient gene transfer. *Virology* 302, 236-244.

Reference to a book:

Hagag, N., Viola, M.V., 1993. Chromosome Microdissection and Cloning: A Practical Guide. Academic Press, San Diego.

Reference to a chapter in an edited book:

Mettam, G.R., Adams, L.B., 1999. How to prepare an electronic version of your article. In: Jones, B.S., Smith, R.Z. (Eds.), Introduction to the Electronic Age. E-Publishing Inc., New York, pp. 281-304.

Citations in the text. Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications should not be in the reference list, but may be mentioned in the text. Citation of a reference as "in press" implies that the item has been accepted for publication.

Citing and listing of Web references. As a minimum, the full URL should be given. Any further information, if known (author names, dates, reference to a source publication, etc.), should also be given.

Digital Object Identifier. Elsevier assigns a unique digital object identifier (DOI) to every article it publishes. The DOI appears on the title page of the article. It is assigned after the article has been accepted for publication and persists throughout the lifetime of the article. Because of its persistence, it can be used to query the publisher for information on the article during the production process, to find the article on the Internet through various Web sites, and to cite the article in academic references. When using an Elsevier article in a reference section, it is important to include the article's DOI in the reference, as volume and page information is not always available for articles published online. When citing a journal article, include the digital object identifier (DOI), if noted, from the article's title page. For example:

Duerst, R.J., Morrison, L.A., 2004. Herpes simplex virus 2 virion host shutoff protein interferes with type I interferon production and responsiveness. *Virology*, doi:10.1016/j.virol.2004.01.019.

DNA sequences and GenBank accession numbers. Authors wishing to enable other scientists to use the accession numbers cited in their papers via links to these sources should type this information in the following manner:

*For each and every accession number cited in an article, authors should type the accession number in **bold, underlined text**. Letters in the accession number should always be capitalized (see example below). This combination of letters and format will enable the typesetter to recognize the relevant texts as accession numbers and add the required link to GenBank's sequences.*

Example: GenBank accession nos. **A1631510**, **A1631511**, **A1632198**, and **BF223228**, a B-cell tumor from a chronic lymphatic leukemia (GenBank accession no. **BE675048**), and a T-cell lymphoma (GenBank accession no. **AA361117**).

Authors are encouraged to check accession numbers used very carefully. **An error in a letter or number can result in a dead link.**

In the final version of the **printed article**, the accession number text will not appear bold or underlined. In the final version of the **electronic copy**, the accession number text will be linked to the appropriate source in the NCBI databases, enabling readers to go directly to that source from the article.

Tables should be separate from the manuscript text and numbered consecutively in accordance with their appearance in the text. Tables can be uploaded individually or consolidated into a single file. The file description you input below when uploading your table must include the table number or range (e.g., Table 1, Tables 2-4). Place footnotes to tables below the table body and indicate them with superscript lowercase letters. Avoid vertical rules. Be sparing in the use of tables and ensure that the data presented in tables do not duplicate results described elsewhere in the article.

Figure legends should contain a brief description of the experiment performed so that the figure can be understood without reference to the body of the text. However, the legend should not repeat Materials and Methods or contain interpretive statements.

Figures. Number the illustrations according to their sequence in the text. Figures intended to be montages of separate panels should be assembled into one figure by the authors. A set of figures, particularly for montages of electron micrographs, can be submitted and labeled as such. Please visit our Web site at <http://authors.elsevier.com/artwork> for detailed instructions on preparing electronic artwork. You are urged to visit this site; some excerpts from the detailed information are given here.

Formats. Regardless of the application used, when your electronic artwork is finalized, please "save as" or convert the images to one of the following formats. (Note the resolution requirements for line drawings, halftones, and line/halftone combinations given below.)

EPS: Vector drawings. Embed the font or save the text as "graphics."

TIFF: Color or gray-scale photographs (halftones): always use a minimum of 300 dpi.

TIFF: Bitmapped line drawings: use a minimum of 1000 dpi.

TIFF: Combinations bitmapped line/halftone (color or gray scale): a minimum of 500 dpi is required.

DOC, XLS, or PPT: If your electronic artwork is created in any of these Microsoft Office applications, please supply "as is."

Please do not:

- Supply embedded graphics in your word processor (spreadsheet, presentation) document.
- Supply files that are optimized for screen use (such as GIF, BMP, PICT, WPG); the resolution is too low.
- Supply files that are too low in resolution.
- Submit graphics that are disproportionately large for the content.

Color figures. One color figure will be published free of charge in each article, provided that color is deemed necessary by the editor. Additional color figures will be charged to the author. However, if together with your accepted article, you submit usable color figures, then Elsevier will ensure, at no additional charge, that these figures will appear in color on the Web (e.g., ScienceDirect and other sites) regardless of whether these illustrations are reproduced in color in the printed version. For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article. For further information on the preparation of electronic artwork, please see <http://authors.elsevier.com/artwork>. [Please note: Because of technical complications that can arise in converting color figures to "gray scale" (for the printed version should you not opt for color in print), please submit in addition usable black-and-white files corresponding to all the color illustrations.]

Preparation of supplementary material. Elsevier now accepts electronic supplementary material to support and enhance your scientific research. Supplementary files offer additional possibilities for publishing supporting applications, movies, animation sequences, high-resolution images, background datasets, sound clips, and more. Supplementary files supplied will be published online alongside the electronic version of your article in Elsevier Web products, including ScienceDirect (<http://www.sciencedirect.com>). To ensure that your submitted material is directly usable, please ensure that data are provided in one of our recommended file formats. Authors should submit the material in electronic format together with the article and supply a concise and descriptive caption for each file. For more detailed instructions please visit our artwork instruction pages at the Author Gateway at <http://authors.elsevier.com/artwork>. For further advice, please contact the Editorial Office (e-mail: virology@elsevier.com; telephone: (619) 699-6394; or fax: (619) 699-6211).

(b) Rapid Communications

Manuscripts in general should be no longer than 12 double-spaced typewritten pages, containing an abstract of approximately 100 words, a one-paragraph introduction, Results, a concise Discussion section, and an abbreviated Materials and Methods section. A maximum of 20 references and four items for the display of data (any combination of figures and tables) will be allowed.

Rapid Communications will be reviewed within 1 to 2 weeks. No significant revisions will be permitted. PDF proofs will be e-mailed to authors. Corrections must be returned within 48 hours. Rapid communications will appear in a special section at the front of the journal. Rapid communications are distinct from the previously published "short communications." This category is not intended for reporting preliminary studies or brief studies of a descriptive nature.

(c) Minireviews

The minireviews will be short (2-4 pages). Inclusion of figures/cartoons to illustrate points is strongly encouraged. The minireview should also direct the reader to a few key papers in the field, which come under the heading "Selected Reading." There is no formal Reference section.

Cover illustrations

Authors are encouraged to submit interesting figures for possible publication on the front cover of an issue of this journal; the figure should be related to the author's article.

MANUSCRIPT CHECKLIST

It is hoped that this list will be useful during the final checking of an article prior to submitting it to the journal for review.

- Ensure that the following items are present:
 - One author designated as corresponding author
 - E-mail address
 - Full postal address
 - Telephone and fax numbers
 - All necessary files have been uploaded
 - Keywords
 - All figure captions
 - All tables (including title, description, footnotes) are provided

Further considerations

- Manuscript has been "spell-checked."
- References are in the correct format for this journal.
- All references mentioned in the Reference list are cited in the text, and vice versa.
- Permission has been obtained for use of copyrighted material from other sources (including the Web).

- Color figures are clearly marked as being intended for color reproduction on the Web (free of charge) and in print or to be reproduced in color on the Web (free of charge) and in black-and-white in print. If only color on the Web is required, black-and-white versions of the figures are also supplied for printing purposes.

For any further information please contact the Author Support Department at authorsupport@elsevier.com.

PROOFS

One set of page proofs in PDF format will be sent by e-mail to the corresponding author, to be checked for typesetting/editing. No changes in, or additions to, the accepted (and subsequently edited) manuscript will be allowed at this stage. Authors are requested to return proof corrections within 72 hours of receipt in order to expedite publication. It is important to ensure that all of your corrections are sent back to us in one communication. Subsequent corrections will not be possible, so please ensure your first sending is complete. Note that this does not mean you have any less time to make your corrections, just that only one set of corrections will be accepted.

[print page](#)[close window](#)[Privacy Policy](#) | [Terms & Conditions](#) | [Contact Us](#)

© 2005 Elsevier Ltd

CANCER MODELS

Systems for Identifying New Drugs Are Often Faulty

Screening potential anticancer drugs sounds easy. Just take a candidate drug, add it to a tumor type of choice, and then monitor whether the agent kills the cells or inhibits cancer growth. Too bad it hasn't been that simple. Even as investigators try to develop a new generation of more effective and less toxic anticancer drugs that directly target the gene changes propelling cells toward uncontrollable division (see p. 1036), they face a long-standing problem: sifting through potential anticancer agents to find ones promising enough to make human clinical trials worthwhile.

Indeed, since formal screening began in 1955, many thousands of drugs have shown activity in either cell or animal models, but only 39 that are used exclusively for chemotherapy, as opposed to supportive care, have won approval from the U.S. Food and Drug Administration. "The fundamental problem in drug discovery for cancer is that the model systems are not predictive at all," says Alan Oliff, executive director for cancer research at Merck Research Laboratories in West Point, Pennsylvania.

Pharmaceutical companies often test drug candidates in animals carrying transplanted human tumors, a model called a xenograft. But not only have very few of the drugs that showed anticancer activity in xenografts made it into the clinic, a recent study conducted at the National Cancer Institute (NCI) also suggests that the xenograft models miss effective drugs. The animals apparently do not handle the drugs exactly the way the human body does. And attempts to use human cells in culture don't seem to be faring any better, partly because cell culture provides no information about whether a drug will make it to the tumor sites.

The pressure is on to do better. So researchers are now trying to exploit recent discoveries about the subtle genetic and cellular changes that lead a cell toward cancer to create cultured cells or animal models that accurately reproduce these changes. "The real challenge for the 1990s is how to maximize our screening systems so that we are using the biological information that has accumulated," says Edward Sausville, associate director of the division of cancer treatment and diagnosis for the developmental therapeutics program at the NCI. "In short, we need to find faithful representations of carcinogenesis."

The first efforts to do so date back to the end of World War II, when hints began

emerging that some chemicals might have cancer-fighting effects. That evidence encouraged many chemists to explore the anticancer potential of similar agents shelved in their laboratories. And after commercial interests decided against helping the academics set up an efficient way to screen their chemicals, the NCI stepped in.

The institute started by pulling together mouse models of three tumors: a leukemia, which affects blood cells; a sarcoma, which arises in bone, muscle, or connective tissue; and a carcinoma, the most common type of cancer, which arises in epithelial cells and includes such major killers as breast, colon, and lung cancers. Initially, many of the agents tested in these models appeared to do well. However, most worked against blood cancers such as leukemia and lymphoma, as opposed to the more common solid tumors. And when tested in human cancer patients, most of these compounds failed to live up to their early promise.

Researchers blamed the failures on the fact that the drugs were being tested against mouse, not human, tumors, and beginning in 1975, NCI researchers came up with the xenograft models, in which investigators implant human tumors underneath the skin of mice with faulty immune systems. Because the animals can't reject the foreign tissue, the tumors usually grow unchecked, unless stopped by an effective drug. But the results of xenograft screening turned out to be not much better than those obtained with the original models, mainly because the xenograft tumors don't behave like naturally occurring tumors in humans—they don't spread to other tissues, for example. Thus, drugs tested in the xenografts appeared effective but worked poorly in humans. "We had basically discovered compounds that were good mouse drugs rather than good human drugs," says Sausville.

The xenograft models may also have

missed effective drugs. When Jacqueline Plowman's team at NCI tested 12 anticancer agents currently used in patients against 48 human cancer cell lines transplanted individually into mice, they found that 30 of the tumors did not show a significant response—defined as shrinking by at least 50%—to any of the drugs.

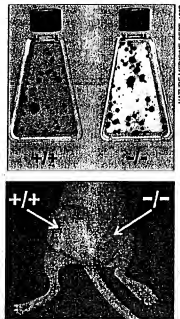
Researchers have not yet figured out why so many of the xenografts were insensitive to the drugs. But the NCI team says that the result means that drugs would have to be screened against six to 12 different xenografts to make sure that no active anticancer drugs were missed. That's an expensive proposition, as the average assay costs about \$1630 when performed by the government and \$2900 when done commercially. "I cannot get on my pulpit and say that the way we are doing this is the best way, because I don't think there is a good way to do it," says Sausville.

To create better models of cancer development in humans, investigators are now drawing on the growing knowledge of human cancer-related gene mutations. They are genetically altering mice so that they carry the same kinds of changes—either abnormal activation of cancer-promoting oncogenes or loss of tumor-suppressor genes—that lead to cancer in humans. The hope is that the mice will develop tumors that behave the same way human tumors do.

So far, the results from these mouse models have been mixed, however. One mutant mouse strain, for example, lacks a working APC gene, a tumor suppressor that leads to colon cancer when lost or inactivated. This mouse seems to do well at re-creating the early signs of colon cancer. But in the later stages of the disease, the type of mutations in the tumors

begin to diverge from those in human colon cancer, and the disease manifests itself differently as well. It spares the liver, for example, unlike the human cancer.

Other new mouse models have fared even worse. Take the one in which the *retinoblastoma* (RB) tumor-suppressor gene was knocked out. In humans, loss of RB leads to a cancer in the retina of the eye. But when the gene is inactivated in mice, the rodents get pituitary gland tumors. And BRCA1 knock-



Not a matched pair. In the clonogenic assay (top), tumor cells with (+/+) and without (-/-) the *p21* gene responded similarly to radiation. But in mice, the *p21*-tumors often shrank, while those having the gene never did.

ous—which are supposed to simulate human breast and ovarian cancer—don't get any tumors at all. "One might expect that these animals would also mimic human symptoms, not just the genetic mutations," says molecular biologist Tyler Jacks of the Massachusetts Institute of Technology. "In fact, that is usually the exception, not the rule."

Why gene knockouts in mice have effects so different from those of the corresponding mutations in humans is unclear. One possibility is that in mice, other genes can compensate for a missing gene, such as *BRCA1*. Another, says Jacks, is that "the genetic wiring for growth control in mice and humans is subtly different."

The limitations of animal models have spurred the NCI, among others, to test drug candidates in cultures of human cells. The institute now relies on a panel of 60 human tumor cell lines, including samples of all the major human malignancies. Drugs to be tested are fed to subsets of the panel, based on tumor cell type, and their cell-killing activity is monitored.

Over the last 7 years, the panel has been used to screen almost 63,000 compounds, and 5000 have exhibited tumor cell-killing activity. But that has created another dilemma, because so many compounds show antitumor cell activity in culture, and the cost of bringing them all to clinical trials—where most don't work anyway—would be daunting. As Sausville asks: "How do you prioritize so many compounds for clinical trials?" For that, the NCI uses a computer database to sift through past antitumor agents and look for only those compounds with novel mechanisms of action. Computer screening has whittled the number of promising agents down to about 1200, according to Sausville.

Those compounds are then tested in what is known as a hollow fiber model, in which tiny tubes filled with tumor cells are implanted into mice in a variety of sites. By monitoring the tumor cell-killing effects of drugs on the implants, researchers can test which drugs actually make it to the tumor sites when the drugs are administered in different ways: intravenously versus orally, for example. Sausville cautions, however, that it's still too early to tell how predictive these screens are, because only a few of the drugs tested have gone far enough to show efficacy in humans.

Both drug screeners and doctors also use another cell culture method, the so-called clonogenic assay, to sift through potential anticancer drugs. They grow cell lines or a patient's tumor cells in petri dishes or culture flasks and monitor the cells' responses to various anticancer treatments. But clono-

genic assays have their problems, too. Sometimes they don't work because the cells simply fail to divide in culture. And the results cannot tell a researcher how anticancer drugs will act in the body.

What's more, new results from Bert Vogelstein's group at Johns Hopkins University School of Medicine add another question mark about the assay's predictive ability. Todd Waldman, a postdoc in the Vogelstein laboratory, found that xenografts and clonogenic assays deliver very different messages about how cancer cells lacking a particular gene, *p21*, respond to DNA-cripping agents.

Radiation, like many of the drugs used to treat cancer, works by damaging the cells' DNA. This either brings cell replication to a halt or triggers a process known as apoptosis in which the cells essentially commit suicide. Waldman wanted to see how *p21*, one of the genes involved in sensing the DNA damage and halting cell replication, influences that response to radiation.

In the mouse xenograft assay, Waldman and his colleagues found that the radiation cured 40% of the tumors composed of cells lacking *p21*, while tumors made of cells carrying the gene were never cured. But this difference was not apparent in the clonogenic assay, where the radiation appeared to thwart the growth of both dispersed tumor cell types. "We showed this gross difference in sensitivity in real tumors in mice and in the clonogenic assay," Waldman says.

He suggests that the different responses in the two systems have to do with the fact that a subset of *p21* mutants die in response to radiation, while cells with the normal gene merely arrest cell division. Either way, the dispersed tumor cells in the clonogenic assay will fail to grow. However, in the xenograft tumors, which consist of many cells in a solid mass, the arrested, but nonetheless living, *p21*⁺ tumor cells may release substances that encourage the growth of any nearby tumor cells that escaped the effects of the radiation. But tumor cells lacking the *p21* gene die, and because dead cells cannot "feed" neighboring

tumor cells, the entire tumor may shrink.

The finding indicates that the clonogenic assay can't always predict how a tumor will respond to a drug in an animal. Still, by linking the different responses in two models to the presence or absence of a specific gene system, the Waldman team's results help clarify why tumor cells might respond differently in culture and in animals. Indeed, the general idea that a tumor's drug sensitivity may be linked to the genetic mutations it carries has led others to try to use cells with comparable mutations to identify better chemotherapeutic agents.

Leland Hartwell, Stephen Friend, and their colleagues at the Fred Hutchinson Cancer Research Center in Seattle are pioneering one such effort. They are building on previous work in which Hartwell's team discovered a series of yeast genes, called checkpoint genes, that normally stop cells from progressing through the cell cycle and dividing if they have abnormalities such as unrepaired DNA damage. Because mutations in checkpoint and other cell cycle-related genes have been linked to human cancers, looking for

drugs that restore normal growth control in mutated yeast might be one way to find new cancer therapies (see Article on p. 1064).

The NCI is taking a similar tack. They are looking to see if they can reclassify the cells in their panel, which was set up based on tissue type—breast cancer versus colon cancer, for example—according to the types of genetic defects the cells carry. To enable drugs that counteract specific defects to be prescribed most effectively, researchers are also developing technologies for analyzing the gene defects in each patient's tumors. That way, if drugs that correct specific defects can be identified, they could then be matched to each individual's tumor cell makeup. "This would be so valuable," says Homer Pearce, vice president of cancer research and clinical investigation at Eli Lilly and Co. in Indianapolis. "It would help to identify patients that have the greatest chance of benefiting from therapy, while minimizing the number that would be exposed to a treatment that would not work."

Indeed, Merck's Oliff says, "the future of cancer drug screening is turning almost exclusively toward defining molecular targets." If the approach works, drug developers would finally have an easy way to identify promising cancer drugs, and cancer patients might have an array of new treatments.

—Trisha Gura

Trisha Gura is a writer in Cleveland, Ohio.

TESTING THE XENOGRAFT ASSAY			
Type of Cancer	Cell Lines Tested	% of Tumors Responding to Minimal Response (< 40% shrinkage)	Significant Response (> 50% shrinkage)
Colon	9	31%	5%
Lung, non-small cell	7	49%	8%
Breast	6	51%	19%
Ovarian	3	35%	0
Renal	5	43%	0

JOURNAL OF VIROLOGY

2005 INSTRUCTIONS TO AUTHORS*

SCOPE

The *Journal of Virology* (JVI) is devoted to the timely dissemination of significant knowledge concerning the viruses of plants, fungi, bacteria, protozoa, and animals. Investigators in areas of basic virology are invited to submit reports of original research that uses the approaches of biochemistry, biophysics, cell biology, epidemiology, genetics, genomics, immunology, molecular biology, morphology, proteomics, physiology, and pathogenesis and immunity. The original articles should contain experimental observations that address a hypothesis, lead to new concepts, and indicate new directions in research. Computational analyses of viruses, virus-like sequences, or viral proteins that advance the field are also appropriate. *The journal will not publish papers that simply provide a new restriction map or nucleotide sequence; identify new immunodominant peptides representing T- or B-cell epitopes; or report the isolation or characterization of monoclonal antibodies, a viral variant, or a new strain or type. Such information or reagents must instead be used in further experimentation to test an idea or relate a clear set of novel conclusions that derive from the data.*

JVI specifically encourages publications relating the viruses under study to their host cells or organisms. In recognition of this emphasis, the sections of the journal relating to viral pathogenesis and immunity and to virus-cell interactions have been specifically set aside and identified in the table of contents. The editors wish to promote the publication of research done at the cell biology-virology-organismic biology interface.

JVI also encourages the submission of manuscripts detailing studies in which viruses or viral genetic elements are used as components of vectors for the delivery of therapeutic genes into animals and plants. These original articles should contain experimental observations that lead to new concepts and understanding relevant to gene delivery, regulated expression of therapeutic genes, or viral pathogenesis. To promote publications in this area, the editors have established a section of the journal for articles relating to gene therapy.

JVI encourages manuscripts that include microarrays and similar parallel profiling analyses of viral or cellular gene expression. However, such manuscripts will be published only if they provide novel insight into the biology of the virus or the infected cell, or if they form the basis for additional experiments that provide such insights. It is expected that the primary data from such analyses will be incorporated into the text or figures or will be made available as supplementary material on the ASM web site, a publicly accessible laboratory website, or a public repository (such as the National Center for Biotechnol-

ogy Information).

ASM publishes a number of journals covering various aspects of microbiology. Each journal has a prescribed scope that must be considered in determining where to publish each manuscript. The following guidelines may be of assistance.

(i) JVI will consider papers that describe the use of antiviral agents in elucidating the basic biological processes of viruses and host cells. Papers dealing with other aspects of antiviral agents and chemotherapy will be considered for *Antimicrobial Agents and Chemotherapy*.

(ii) JVI will consider all papers dealing with the biology of bacteriophages. Studies involving the use of bacteriophages as a diagnostic typing system will be considered by the *Journal of Clinical Microbiology*. Those dealing with phages in relation to industrial microbiology will be considered by *Applied and Environmental Microbiology*.

(iii) Manuscripts describing new methods or improvements in media and culture conditions will not be considered by JVI unless the procedures are applied to the study of basic problems in virology or cell biology. Such manuscripts are more appropriate for *Applied and Environmental Microbiology* or the *Journal of Clinical Microbiology*. By the same token, manuscripts dealing with methods for the production of monoclonal antibodies will not be considered unless the methods have been used to address fundamental questions.

(iv) Manuscripts dealing with clinical investigations, excluding those concerned with the activities of antiviral agents, should be submitted to the *Journal of Clinical Microbiology*. Manuscripts dealing with ecology or environmental studies are more appropriate for *Applied and Environmental Microbiology*.

Questions about these guidelines may be directed to the editor in chief of the journal being considered.

If transfer to another ASM journal is recommended by an editor, the corresponding author will be contacted.

Note that a manuscript rejected by one ASM journal on scientific grounds or on the basis of its general suitability for publication is considered rejected by all other ASM journals.

EDITORIAL POLICY

Use of Microbiological Information

The Council Policy Committee (CPC) of the American Society for Microbiology affirms the long-standing position of the Society that microbiologists will work for the proper and beneficent application of science and will call to the attention of the public or the appropriate authorities misuses of microbiology or of information derived from microbiology. ASM members are obligated to discourage any use of microbiology contrary to the welfare of humankind, including the use of microbes as

* Shading indicates material that has been added or updated.

biological weapons. Bioterrorism violates the fundamental principles expressed in the Code of Ethics of the Society and is abhorrent to ASM and its members.

ASM recognizes that there are valid concerns regarding the publication of information in scientific journals that could be put to inappropriate use as described in the CPC resolution mentioned above. Members of the ASM Publications Board will evaluate the rare manuscript that might raise such issues during the review process. However, as indicated elsewhere in these Instructions, research articles must contain sufficient detail, and material/information must be made available, to permit the work to be repeated by others. Supply of materials should be in accordance with laws and regulations governing the shipment, transfer, possession, and use of biological materials and must be for legitimate, bona fide research needs. Links to, and information regarding, these laws and regulations can be found at <http://www.asm.org/Policy/index.asp?bid=52>.

General Requirements

Manuscripts submitted to the journal must represent reports of original research, and the original data must be available for review by the editor if necessary.

All authors of a manuscript must have agreed to its submission and are responsible for its content, including appropriate citations and acknowledgments, and must also have agreed that the corresponding author has the authority to act on their behalf in all matters pertaining to publication of the manuscript. The corresponding author is responsible for obtaining such agreements and for informing the coauthors of the manuscript's status throughout the submission, review, and publication process. For Authors' Corrections and Retractions, signed letters of agreement from all of the authors must be submitted (see p. 10–11).

By submission of a manuscript to the journal, the authors guarantee that they have the authority to publish the work and that the manuscript, or one with substantially the same content, was not published previously, is not being considered or published elsewhere, and was not rejected on scientific grounds by another ASM journal.

It is expected that the authors will provide written assurance that permission to cite unpublished data or personal communications has been granted.

By publishing in the journal, the authors agree that any plasmids, viruses, and living materials such as microbial strains and cell lines newly described in the article are available from a national collection or will be made available in a timely fashion and at reasonable cost to members of the scientific community for non-commercial purposes.

Primary Publication

A scientific paper or its substance published in a serial, periodical, book, conference report, symposium proceeding, or technical bulletin, posted on a nonpersonal

website, or made available through any other retrievable source, including CD-ROM and other electronic forms, is unacceptable for submission to an ASM journal on grounds of prior publication.

Posting of a method/protocol on a nonpersonal website should not interfere with the author's ability to have a manuscript utilizing that technique considered for publication in an ASM journal; however, ultimately, it is an editorial decision whether the method constitutes the substance of a paper.

Posting of a limited amount of original data on a personal/university/company website or websites of small collaborative groups working on a problem does not preclude subsequent submission to, and publication by, an ASM journal. The posted data, however, may not constitute the substance of the submission. Specific questions about this policy may be referred to the Publications Board chairman on a case-by-case basis. Posting of theses and dissertations on a personal/university-hosted website does not preclude subsequent submission to, and publication by, an ASM journal.

Posting of unpublished sequence data on the Internet is usually not considered prior publication; however, the address (URL) of the source of the sequence should be included in the text.

Preliminary disclosures of research findings, webcast as meeting presentations or published in abstract form as adjuncts to a meeting, e.g., part of a program, are not considered prior publication.

It is incumbent upon the author to acknowledge any prior publication of the data contained in a manuscript submitted to an ASM journal. A copy of the relevant work should be submitted with the paper as supplemental material.

Ultimately, it is an editorial decision whether the material constitutes the substance of a paper.

Permissions

The corresponding author is responsible for obtaining permission from both the original author and the original publisher (i.e., the copyright owner) to reproduce or modify figures and tables and to reproduce text (in whole or in part) from previous publications.

The original signed permission(s) must be submitted directly to the editor, outside the Rapid Review system, no later than the modification stage and should be identified as to the relevant item in the ASM manuscript (e.g., "permissions for Fig. 1 in JVI00123-05"). In addition, a statement indicating that the material is being reprinted with permission must be included in the relevant figure legend or table footnote of the manuscript. Reprinted text must be enclosed in quotation marks, and the permission statement must be included as running text or indicated parenthetically.

For supplemental material intended for posting by ASM (see p. 5), if the authors of the JVI manuscript are not also the owner of the supplemental material, the corresponding author must send to ASM signed permission from the copyright owner that allows posting of the

material, as a supplement to the article, by ASM. The corresponding author is also responsible for incorporating in the supplemental material any copyright notices required by the owner.

Authorship

An author is one who made a substantial contribution to the overall design and execution of the experiments; therefore, **ASM considers all authors responsible for the entire paper.** Individuals who provided assistance, e.g., supplied strains or reagents or critiqued the paper, need not be listed as authors but may be recognized in the Acknowledgments section.

A study group, surveillance team, working group, consortium, or the like (e.g., the Active Bacterial Core Surveillance Team) may be listed as a coauthor in the byline if its contributing members satisfy the requirements for authorship and accountability as described in these Instructions. The names (and institutional affiliations if desired) of the contributing members only should be listed in a separate paragraph in the Acknowledgments section. (A footnote directing readers from the group's name in the byline to the contributing individuals' names in the Acknowledgments section will be added by the ASM editorial staff.)

If the contributing members of the group associated with the work do not fulfill the criteria of substantial contribution to and responsibility for the paper, the group may not be listed in the author byline. Instead, it and the names of its contributing members may be listed in the Acknowledgments section.

All authors must agree to the order in which their names are listed in the byline. Statements regarding equal contributions by two or more authors (e.g., X.J. and Y.S. contributed equally to ...) are permitted as footnotes to bylines. Other statements of attribution may be included in the Acknowledgments section.

A change in authorship (order of listing or addition or deletion of a name) **after submission of the manuscript will be implemented only after receipt of signed statements of agreement from all parties involved.** Disputes about authorship may delay review and/or publication of the manuscript.

Conflict of Interest

All authors are expected to disclose, in the manuscript submittal letter, any commercial affiliations as well as consultancies, stock or equity interests, and patent-licensing arrangements that could be considered to pose a conflict of interest regarding the submitted article. (Inclusion of a company name in the author address lines of the manuscript does not constitute disclosure.) Details of the disclosure to the editor will remain confidential. However, it is the responsibility of authors to provide, in the Acknowledgments section, a *general* statement disclosing financial or other relationships that are relevant to the study. Examples of potentially conflicting interests that should be disclosed include relationships that might detract from an

author's objectivity in presentation of study results, and interests whose value would be enhanced by the results presented. All funding sources for the project, institutional and corporate, should be credited in the Acknowledgments section, as described below. In addition, if a manuscript concerns a commercial product, the manufacturer's name must be indicated in the Materials and Methods section or elsewhere in the text, as appropriate, in an obvious manner.

Copyright

To maintain and protect the Society's ownership and rights and to continue to afford scientists the opportunity to publish in high-quality journals, ASM requires the corresponding author to sign a copyright transfer agreement on behalf of all the authors. This agreement is sent to the corresponding author when the manuscript is accepted and scheduled for publication. Unless this agreement is executed (*without changes and/or addenda*), ASM will not publish the manuscript.

In the copyright transfer agreement signed by an author, ASM grants to that author (and coauthors) the right to republish *discrete portions* of his (their) article in any other publication (print, CD-ROM, and other electronic forms) of which he is (they are) the author(s) or editor(s), *on the condition that appropriate credit is given to the original ASM publication.* This republication right also extends to posting on a host computer to which there is access via the Internet. Except as indicated below, significant portions of the article may *not* be reprinted/posted without ASM's prior written permission, however, as this would constitute duplicate publication.

Authors may post their own published articles on their *personal or university-hosted* (but not corporate, government, or similar) websites without ASM's prior written permission provided that appropriate credit is given (i.e., either the copyright lines shown on the top of the first page of the PDF version or "Copyright © American Society for Microbiology, [insert journal name, volume number, page numbers, and year]" for the HTML version).

The copyright transfer agreement asks that authors who were U.S. Government employees and who wrote the article as part of their employment duties be identified. This is because works authored solely by such U.S. Government employees are not subject to copyright protection, so there is no copyright to be transferred. The other provisions of the copyright transfer agreement, such as author representations of originality and authority to enter into the agreement, apply to U.S. Government employee-authors as well as to other authors.

ASM also requires that copyright transfer agreements be signed for cover artwork/photographs.

Copyright for supplemental material (see p. 5) remains with the author, but a license permitting the posting by ASM will be sent, along with the article copyright transfer agreement, to the corresponding author for signing at the acceptance stage. (If the author of the article is not also the copyright owner of the supplemental material, the corresponding author must send to

ASM signed permission from the owner that allows posting of the material, as a supplement to the article, by ASM. The corresponding author is also responsible for incorporating into the supplemental material any copy-right notices required by the owner.)

Use of Human Subjects or Animals in Research

The use of human subjects or other animals for research purposes is regulated by the federal government and individual institutions. Manuscripts containing information related to human or animal use should clearly state that the research has complied with all relevant federal guidelines and institutional policies. Copies of these guidelines and policy statements must be available for review by the editor if necessary.

Patient Identification

When isolates are derived from patients in clinical studies, do not identify them by using the patients' initials, even as part of a strain designation. Change the initials to numerals or use randomly chosen letters. Do not give hospital unit numbers; if a designation is needed, use only the last two digits of the unit. (Note: Established designations of some viruses and cell lines, although they consist of initials, are acceptable [e.g., JC virus, BK virus, and HeLa cells].)

Nucleotide and Amino Acid Sequences

It is expected that newly determined nucleotide and/or amino acid sequence data will be deposited and GenBank/EMBL/DBJ accession numbers will be included in the manuscript no later than the modification stage of the review process. It is also expected that the sequence data will be released to the public no later than the publication date of the article. The accession number should be included in a separate paragraph at the end of the Materials and Methods section for full-length papers or at the end of the text for Notes. If conclusions in a manuscript are based on the analysis of sequences and a GenBank/EMBL/DBJ accession number is not provided at the time of the review, authors should provide the sequence data as supplemental material.

It is expected that, when previously published sequence accession numbers are cited in a manuscript, the original citations (e.g., journal articles) will be included in the References section when possible or reasonable.

Authors are also expected to do elementary searches and comparisons of nucleotide and amino acid sequences against the sequences in standard databases (e.g., GenBank) immediately before manuscripts are submitted and again at the proof stage.

Analyses should specify the database, and the date of each analysis should be indicated in the format MM/YY. If relevant, the version of the software used should be specified.

Database address information is as follows:

DDBI: Center for Information Biology and DNA

Data Bank of Japan, National Institute of Genetics, 1111 Yata, Mishima, Shizuoka 411-8540, Japan; telephone, 81-559-81-6853; fax, 81-559-81-6849; e-mail, ddbj@ddbj.nig.ac.jp (for data submissions); URL, <http://www.ddbj.nig.ac.jp>.

EMBL: EMBL Nucleotide Sequence Submissions, European Bioinformatics Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SD, United Kingdom; telephone, 44-1223-494499; fax, 44-1223-494472; e-mail, datasubs@ebi.ac.uk; URL, <http://www.ebi.ac.uk>.

GenBank: National Center for Biotechnology Information, National Library of Medicine, Bldg. 38A, Rm. 8N-803, Bethesda, MD 20894; telephone, 301-496-2475; fax, 301-480-9241; e-mail, info@ncbi.nlm.nih.gov; URL, <http://www.ncbi.nlm.nih.gov>.

See p. 13 for nucleic acid sequence formatting instructions.

Structural Determinations

It is expected that coordinates for new structures of macromolecules will be deposited in the Protein Data Bank and that assigned identification codes will be included in the manuscript no later than the modification stage of the review process. It is also expected that the coordinates will be released to the public no later than the publication date of the article. Authors are encouraged to send coordinates with their original submission, however, so that reviewers can examine them along with the manuscript. The accession number(s) should be listed in a separate paragraph at the end of the Materials and Methods section for full-length papers or at the end of the text for Notes. The URLs for coordinate deposition are <http://pdb.rutgers.edu> and <http://pdbdep.protein.osaka-u.ac.jp>.

Compliance

Failure to comply with the policies described in these Instructions may result in a letter of reprimand, a suspension of publishing privileges in ASM journals, and/or notification of the authors' institutions.

Warranties and Exclusions

Articles published in this journal represent the opinions of the authors and do not necessarily represent the opinions of ASM. ASM does not warrant the fitness or suitability, for any purpose, of any methodology, kit, product, or device described or identified in an article. The use of trade names is for identification purposes only and does not constitute endorsement by ASM.

Page Charges

Authors whose research was supported by grants, special funds (including departmental and institutional), or contracts (including governmental) or whose research was done as part of their official duties (government, corporate, etc.) are required to pay page charges.

For a **corresponding author who is an ASM member**, page charges are currently \$85 per page for the first seven pages and \$140 per page for each page in excess of seven (subject to change without notice). To obtain the member rate, the *corresponding author* must be an ASM member and must include his member number on the reprint/page charge form.

For a **nonmember corresponding author**, page charges are currently \$98 per page for the first seven pages and \$161 for each page in excess of seven. A corresponding author who is not an ASM member may join ASM to obtain the member rate by completing the ASM member application form sent with the acceptance letter and returning it along with payment and the completed reprint/page charge form to the Journals Department.

If the research was not supported by any of the means described above, a request to waive the charges may be mailed (Journals Department, ASM, 1752 N St., N.W., Washington, DC 20036-2904, USA) or faxed (202-942-9355) to the Journals Department. This request must indicate how the work was supported and should be accompanied by copies of the title page and Acknowledgments section.

Minireviews, Guest Commentaries, and Comment Letters to the Editor are not subject to page charges. New-Data Letters to the Editor are subject to page charges.

Supplemental Material

Supplemental material intended for posting by ASM may not simply include additional figures or tables that support the authors' conclusions. It must be restricted to large or complex data sets or results that cannot be readily displayed in printed form because of space or technical limitations. Such material may include data from microarray, structural, biochemical, or video imaging analyses. In such cases, the manuscript submitted for review should include a distillation of the results so that the principal conclusions are fully supported without referral to the supplemental material.

Supplemental material intended for posting by ASM must be uploaded where indicated on the Rapid Review submission form and will be reviewed along with the manuscript. The decision to publish (i.e., post online only) the material with the article if it is accepted will be made by the editor and conveyed to the corresponding author in the acceptance e-mail. Note, therefore, it is possible that an article will be accepted but that the supplemental material will not be.

If the software required for users to view/use the supplemental material is not embedded in the file, you are urged to use shareware or generally available/easily accessible programs.

Unlike the article, supplemental material will not be edited by the ASM Journals staff and "proofs" will not be made available.

Supplemental material will always remain associated with its article and is not subject to any modifications

after publication.

Material that has been published previously (print or online) is not acceptable for posting as supplemental data. Instead, the appropriate reference(s) to the original publication should be made in the article text.

Copyright for the supplemental material remains with the author, but a license permitting the posting by ASM will be sent, along with the article copyright transfer agreement, to the corresponding author for signing. If you are not the copyright owner, you must provide to ASM signed permission from the owner that allows posting of the material, as a supplement to your article, by ASM. You are responsible for including in the supplemental material any copyright notices required by the owner.

A one-time charge (amount not yet determined) may be levied for posting of supplemental material. When instituted, the charge will be indicated in the ASM acceptance letter and on the page charge/reprint form.

Editorial Style

The editorial style of ASM journals conforms to the *ASM Style Manual for Journals* (American Society for Microbiology, 2005, in-house document) and *How To Write and Publish a Scientific Paper*, 5th ed. (Oryx Press, 1998), as interpreted and modified by the editors and the ASM Journals Department.

The editors and the Journals Department reserve the privilege of editing manuscripts to conform with the stylistic conventions set forth in the aforesaid publications and in these Instructions.

Review Process

All manuscripts are considered to be confidential and are reviewed by the editors, members of the editorial board, or qualified ad hoc reviewers. To expedite the review process, authors should recommend at least two reviewers who are not members of their institution(s) and have never been associated with them or their laboratory(ies); please provide their contact information where indicated on the submission form.

Copies of in-press and submitted manuscripts that are important for judgment of the present manuscript should be included as supplemental material to facilitate the review.

When a manuscript is submitted to the journal, it is given a number (e.g., JVI00047-05 version 1) and assigned to one of the editors. (Always refer to this number in communications with the editor and the Journals Department.) It is the responsibility of the corresponding author to inform the coauthors of the manuscript's status throughout the submission, review, and publication processes. The reviewers operate under strict guidelines set forth in "Guidelines for Reviewers" (<http://www.journals.asm.org/misc/reviewguide.shtml>) and are expected to complete their reviews expeditiously.

The corresponding author is notified, generally within 4 to 6 weeks after submission, of the editor's decision to

accept, reject, or require modification. When modification is requested, the corresponding author must either submit the modified version within 2 months or withdraw the manuscript. A point-by-point response to the reviews must be provided in the Rebuttal section of the Rapid Review submission form for the revised manuscript, and a compare copy of the manuscript (without figures) should be included as supplemental material if the editor requested one.

Manuscripts that have been rejected, or withdrawn after being returned for modification, may be resubmitted if the major criticisms have been addressed. (Note: A manuscript rejected by one ASM journal on scientific grounds or on the basis of its general suitability for publication is considered rejected by all other ASM journals.) The cover letter must state that the manuscript is a resubmission, and the *former manuscript number should be provided* in the appropriate field on the submission form. A point-by-point response to the reviews and a compare copy of the revised manuscript showing the changes should be included as supplemental material (the Rebuttal section appears in the submission form only if the manuscript is a modification). Resubmitted manuscripts are normally handled by the original editor.

Manuscripts may not be resubmitted more than once unless permission has been obtained from the original editor or from the editor in chief.

Notification of Acceptance

When an editor has decided that a manuscript is acceptable for publication on the basis of scientific merit, the author and the Journals Department are notified. The text files undergo an automated preediting, cleanup, and tagging process specific to the particular article type, and the illustrations are examined. If all files have been prepared according to the criteria set forth in these Instructions and those in *Rapid Review*, the acceptance procedure will be completed successfully. If there are problems that would cause extensive corrections to be made at the copyediting stage or if the files are not acceptable for production, the ASM production editor will contact the corresponding author.

Once all the material intended for publication has been determined to be adequate, the manuscript is scheduled for the next available issue and an acceptance letter indicating the month of publication, approximate page proof dates, and table of contents section is mailed to the corresponding author; a copyright transfer agreement is also included, as is a license to permit posting of supplemental material (if applicable). The editorial staff of the ASM Journals Department completes the editing of the manuscript to bring it into conformity with prescribed standards.

Page Proofs

Page proofs, together with a query sheet, a reprint order/page charge form, and instructions for handling proofs, will be made available to the corresponding author

electronically via a PDF file that can be accessed through a unique password. Since corresponding authors will be notified of the availability of their PDF proofs and assigned their unique password via e-mail, an e-mail address **must be supplied** in the correspondent footnote. Failure to do so may result in a delay in publication. **The PDF page proofs must be printed out, and corrections must be written on the hard copy.** Queries must be answered on the query page or on a separate sheet of paper, and any changes related to the queries must be indicated on the proofs. Note that the copy editor does not query at every instance where a change has been made. Queries are written only to request necessary information or clarification of an unclear passage or to draw attention to edits that may have altered the sense. It is the author's responsibility to read the entire text, tables, and figure legends, not just items queried. As soon as the page proofs are corrected and signed by the person who proofread them (within 48 h), **they should be mailed or sent by a courier service such as FedEx, not faxed or sent as an e-mail attachment, to the ASM Journals Department.**

The proof stage is not the time to make extensive corrections, additions, or deletions. Important new information that has become available between acceptance of the manuscript and receipt of the proofs may be inserted as an addendum in proof with the permission of the editor. If references to unpublished data or personal communications are added, it is expected that written assurance granting permission for the citation will be included. Limit changes to correction of spelling errors, incorrect data, and grammatical errors and updated information for references to manuscripts that have been submitted or are in press. If URLs have been provided in the article, recheck the sites to ensure that the addresses are still accurate and the material that you expect the reader to find is indeed there.

Questions about *late proofs and problems in the proofs* should be directed to the ASM Journals Department (telephone, 202-942-9234). Questions about *accessing or viewing your PDF proofs* should be directed to Katie Gay of Cadmus Professional Communications at 804-261-3155 or gayk@cadmus.com.

Reprints

Reprints (in multiples of 100) may be purchased by all coauthors. An order form that includes a table showing the cost of reprints is sent with the proof to the corresponding author.

The corresponding authors of Minireviews and Guest Commentaries will receive 100 free reprints of their contribution; additional reprints (in multiples of 100) may be purchased if desired. As for regular articles, an order form showing the cost of reprints will be sent with the proofs.

PDF Files

A corresponding author who has included an e-mail address in his "corresponding author" footnote will have

limited access (10 downloads, total) to the PDF file of his published article. An e-mail alert will automatically be sent to him on the day the issue is posted. It will provide a URL, which will be required to obtain access, and instructions. An article may be viewed, printed, or stored, provided that it is for the author's own use.

Should coauthors or colleagues be interested in viewing the paper for their own use, the corresponding author may provide them with the URL; a copy of the article may not be forwarded electronically. However, they must be made aware of the terms and conditions of the ASM copyright. (For details, go to <http://www.journals.asm.org/misc/terms.shtml>.) Note that each such download will count toward the corresponding author's total of 10. After 10 downloads, access will be denied and can be obtained only through a subscription to the journal (either individual or institutional) or after the standard access control has been lifted (i.e., 6 months after publication).

HOW TO SUBMIT MANUSCRIPTS

All submissions to JVI must be made electronically via the Rapid Review online submission and peer review system at the following URL: www.rapidreview.com/ASM2/author.html. (E-mailed submissions will not be accepted.) First-time users must create an Author account, which may be used for submitting to all ASM journals. Instructions for creating an Author account are available at the above URL under the New to Rapid Review? button. The person from whose account a submission is made will be recorded as the corresponding author, and any correspondence will be sent to the e-mail address in that account. Step-by-step instructions for submitting a manuscript via Rapid Review are available from the account holder's My Manuscripts page. Information on file types acceptable for electronic submission can be found under the More About File Formats button.

ORGANIZATION AND FORMAT

On receipt at ASM, an accepted manuscript undergoes an automated preediting, cleanup, and tagging process specific to the particular article type. To optimize this process, manuscripts must be supplied in the correct format and with the appropriate sections and headings.

Type every portion of the manuscript double spaced (a minimum of 6 mm between lines), including figure legends, table footnotes, and References, and number all pages in sequence, including the abstract, figure legends, and tables. Place the last two items after the References section. Manuscript pages should have line numbers. The font size should be no smaller than 12 pt. It is recommended that the following sets of characters be easily distinguishable in the manuscript: the numeral zero (0) and the letter "oh" (O); the numeral one (1), the letter "el" (l), and the letter "eye" (I); and a multi-

plication sign (×) and the letter "ex" (x). Do not create symbols as graphics or use special fonts that are external to your word processing program; use the "insert symbol" function. Set the page size to 8½ by 11 inches (ca. 21.6 by 28 cm). Italicize or underline any words that should appear in italics, and indicate paragraph lead-ins in bold type.

Authors who are unsure of proper English usage should have their manuscripts checked by someone proficient in the English language. **Manuscripts may be editorially rejected, without review, on the basis of poor English or lack of conformity to the standards set forth in these Instructions.**

Full-Length Papers

Full-length papers should include the elements described in this section.

Title, running title, and byline. Each manuscript should present the results of an independent, cohesive study; thus, numbered series titles are not allowed. Avoid the main title/subtitle arrangement, complete sentences, and unnecessary articles. On the title page, include the title, running title (not to exceed 54 characters and spaces), name of each author, address(es) of the institution(s) at which the work was performed, each author's affiliation, and a footnote indicating the present address of any author no longer at the institution where the work was performed. Place an asterisk after the name of the author to whom inquiries regarding the paper should be addressed (see "Correspondent footnote" below).

Also include on the title page the word count for the abstract and the word count for the text (excluding the references, table footnotes, and figure legends).

Study group in byline. A study group, surveillance team, working group, consortium, or the like (e.g., the Active Bacterial Core Surveillance Team) may be listed as a coauthor in the byline if its contributing members satisfy the requirements for authorship and accountability as described in these Instructions. The names (and institutional affiliations if desired) of the contributing members only should be listed in a separate paragraph in the Acknowledgments section. (A footnote directing readers from the group's name in the byline to the contributing individuals' names in the Acknowledgments section will be added by the ASM editorial staff. The list of study group members may *not* be provided in a footnote to the byline, in the text, or in an Appendix.)

If the contributing members of the group associated with the work do not fulfill the criteria of substantial contribution to and responsibility for the paper, the group may not be listed in the author byline. Instead, it and the names of its contributing members may be listed in the Acknowledgments section.

Correspondent footnote. The complete mailing address, a single telephone number, a single fax number,

and a single e-mail address for the corresponding author should be included on the title page of the manuscript. This information will be published in the article as a footnote to facilitate communication, and the e-mail address will be used to notify the corresponding author of availability of proofs and, later, of the PDF file of the published article.

Abstract. Limit the abstract to **250 words or fewer** and concisely summarize the basic content of the paper without presenting extensive experimental details. Avoid abbreviations and references, and do not include diagrams. When it is essential to include a reference, use the same format as shown for the References section but omit the article title. Because the abstract will be published separately by abstracting services, it must be complete and understandable without reference to the text.

Introduction. The introduction should supply sufficient background information to allow the reader to understand and evaluate the results of the present study without referring to previous publications on the topic. The introduction should also provide the hypothesis that was addressed or the rationale for the present study. Choose references carefully to provide the most salient background rather than an exhaustive review of the topic.

Materials and Methods. The Materials and Methods section should include sufficient technical information to allow the experiments to be repeated. When centrifugation conditions are critical, give enough information to enable another investigator to repeat the procedure: make of centrifuge, model of rotor, temperature, time at maximum speed, and centrifugal force ($\times g$ rather than revolutions per minute). For commonly used materials and methods (e.g., media and protein concentration determinations), a simple reference is sufficient. If several alternative methods are commonly used, it is helpful to identify the method briefly as well as to cite the reference. For example, it is preferable to state "cells were broken by ultrasonic treatment as previously described (9)" rather than to state "cells were broken as previously described (9)." The reader should be allowed to assess the method without constant reference to previous publications. Describe new methods completely and give sources of unusual chemicals, equipment, or microbial strains. When large numbers of microbial strains or mutants are used in a study, include tables identifying the immediate sources (i.e., sources from whom the strains were obtained) and properties of the strains, mutants, bacteriophages, plasmids, etc.

A method, strain, etc., used in only one of several experiments reported in the paper may be described in the Results section or very briefly (one or two sentences) in a table footnote or figure legend. It is expected that the sources from whom the strains were obtained will be identified.

Results. In the Results section, include the rationale

or design of the experiments as well as the results; reserve extensive interpretation of the results for the Discussion section. Present the results as concisely as possible in **one** of the following: text, table(s), or figure(s). Data in tables (e.g., cpm of radioactivity) should not contain more significant figures than the precision of the measurement allows. Illustrations (particularly photomicrographs and electron micrographs) should be limited to those that are absolutely necessary to show the experimental findings. Number figures and tables in the order in which they are cited in the text, and be sure to cite all figures and tables.

Discussion. The Discussion should provide an interpretation of the results in relation to previously published work and to the experimental system at hand and should not contain extensive repetition of the Results section or reiteration of the introduction. In short papers, the Results and Discussion sections may be combined.

Acknowledgments. The source of any financial support received for the work being published must be indicated in the Acknowledgments section. (It will be assumed that the absence of such an acknowledgment is a statement by the authors that no support was received.) The usual format is as follows: "This work was supported by Public Health Service grant CA-01234 from the National Cancer Institute."

Recognition of personal assistance should be given as a separate paragraph, as should any statements disclaiming endorsement or approval of the views reflected in the paper or of a product mentioned therein.

References. (i) **Works listed in References.** The References section must include all journal articles (both print and online), books and book chapters (both print and online), patents, theses and dissertations, and published conference proceedings (not abstracts; see below), as well as in-press journal articles, book chapters, and books (publication title must be given). All listed references must be cited in the text. Arrange the citations in **alphabetical order** (letter by letter, ignoring spaces and punctuation) by first author and **number consecutively**. Provide the names of all the authors for each reference. Since title and byline information that is downloaded from PubMed does not show accents, italics, or special characters, authors should refer to the PDF files or hard-copy versions of the articles and incorporate the necessary corrections in the submitted manuscript. Abbreviate journal names according to *Biosis Serial Sources* (BIOSIS, Philadelphia, Pa., 2004). Cite each listed reference by number in the text.

Follow the styles shown in the examples below.

Print references:

1. Arendsen, A. F., M. Q. Solimar, and S. W. Ragsdale. 1999. Nitrate-dependent regulation of acetate biosynthesis and nitrate respiration by *Clostridium thermoaceticum*. *J. Bacteriol.* **181**:1489-1495.

2. Cox, C. S., B. R. Brown, and J. C. Smith. J. Gen. Genet., in press. * *{Article title is optional; journal title is mandatory.}*
3. De Ley, J., M. Gillis, and J. Swings. 1984. Family VI. *Acetobacteraceae* Gillis and De Ley 1980, 23^{pp}, p. 267–278. In N. R. Krieg and J. G. Holt (ed.), *Bergey's manual of systematic bacteriology*, vol. 1. Williams & Wilkins, Baltimore, Md.
4. Dunne, W. M., Jr., F. S. Nolte, and M. L. Wilson. 1997. Cumitech 1B, Blood cultures III. Coordinating ed., J. A. Hindler. American Society for Microbiology, Washington, D.C.
5. Fitzgerald, G., and D. Shaw. In A. E. Waters (ed.), *Clinical microbiology*, in press. EFH Publishing Co., Boston, Mass. * *{Chapter title is optional.}*
6. Gershon, A. A., P. LaRussa, and S. P. Steinberg. 1999. Varicella-zoster virus, p. 900–911. In P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Tenover (ed.), *Manual of clinical microbiology*, 7th ed. American Society for Microbiology, Washington, D.C.
7. Green, P. N., D. Hood, and C. S. Dow. 1984. Taxonomic status of some methylotrophic bacteria, p. 251–254. In R. L. Crawford and R. S. Hanson (ed.), *Microbial growth on C₁ compounds*. Proceedings of the 4th International Symposium. American Society for Microbiology, Washington, D.C.
8. Odell, J. C. 1970. Process for batch culturing. U.S. patent 484,363,770. *{Include the name of the patented item/process if possible.}*
9. O'Malley, D. R. 1998. Ph.D. thesis. University of California, Los Angeles. *{Title is optional.}*

* A reference to an in-press ASM publication should state the control number (e.g., JVI00577-05) if it is a journal article or the name of the publication if it is a book.

Online references:

1. Dimick, J. B., H. G. Welch, and J. D. Birkmeyer. 18 August 2004, posting {or revision} date. Surgical mortality as an indicator of hospital quality. JAMA 292. [Online.] <http://jama.ama-assn.org/cgi/content/short/292/7/847>. *{For online journals; page numbers may not be available.}*
2. Sullivan, C. J. (ed.). 1999–2001. Fungi: an evolving electronic resource for the microbiological community. ASM Press. [Online.] <http://link.asmsusa.de/link/service/books/91090>. Accessed 7 September 2001. *{For online-only books.}*
3. Zellnitz, F., and P. M. Foley. 2 October 1998, posting {or revision} date. History of virology. Am. Virol. J. 1:30–50. [Online.] <http://www.vj.html>. *{For online-only journals; page numbers may not be available.}*
4. Zheng, Z., and J. Zou. 5 September 2001. The initial step of the glycerolipid pathway: identification of glycerol-3-phosphate/dihydroxyacetone phosphate dual substrate acyltransferases in *Saccharomyces cerevisiae*. J. Biol. Chem. doi:10.1074/jbc.M104749200. *{For papers published online in manuscript form.}*

NOTE: A URL or DOI is necessary for each online-only reference; a posting or accession date is required for any online reference that is periodically updated or changed.

(ii) **Items cited in the text.** References to unpublished data, articles submitted for publication, meeting abstracts (including those published in journal supplements), personal communications, letters (irrespective of type) and authors' replies to letters, company publications, patent applications and patents pending, computer software, databases, and websites should be made parenthetically in the text as follows.

...similar results (R. B. Layton and C. C. Weathers, unpublished data).

...system was used (J. L. McInerney, A. F. Holden, and P. N. Brighton, submitted for publication).

...in mitochondria (S. De Wit, C. Thioux, and N. Clumeck, Abstr. 34th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 114, 1994).

...for other bacteria (A. X. Jones, personal communication).

...discussed previously (L. B. Jensen, A. M. Hammerum, R. L. Poulsen, and H. Westh, Letter, Antimicrob. Agents Chemother. 43:724–725, 1999).

...discussed previously (S. L. W. On and P. A. R. Vandamme, Authors' Reply to Letter, J. Clin. Microbiol. 39:2751–2752, 2001).

...the manufacturer (Sigma manual, Sigma Chemical Co., St. Louis, Mo.).

...this process (V. R. Smoll, 20 June 1999, Australian Patent Office). *{For non-U.S. patent applications, give the date of publication of the application.}*

...information found at the XYZ website (http://cbx_iou.pgr).

...the ABC program (version 2.2; Department of Microbiology, State University [<http://www.stu.micro>]).

URLs for companies that produce any of the products mentioned in your study or for products being sold may NOT be included in the article. However, company URLs that permit access to scientific data related to the study or to shareware used in the study are permitted.

Notes

The Note format is intended for the presentation of brief observations that do not warrant full-length papers. Submit Notes in the same way as full-length papers. They receive the same review, they are not published more rapidly than full-length papers, and they are not considered preliminary communications.

Each Note must have an abstract of no more than 100 words. Do not use section headings in the body of the

Note; combine methods, results, and discussion in a single section. Paragraph lead-ins are permissible. The text should be kept to a minimum and **should not exceed 1,200 words**; the total number of figures and tables should not exceed four. **Materials and methods should be described in the text, not in figure legends or table footnotes.** Present acknowledgments as in full-length papers, but do not use a heading. The References section is identical to that of full-length papers.

Minireviews

Minireviews are brief (**limit of 6 printed pages exclusive of references**) summaries of important developments in virology research. They must be based on published articles and may address any subject within the scope of the journal.

Minireviews are solicited by the Minireview editor and are subject to review. Unsolicited reviews will not be considered. Ideas for Minireviews may be sent to the Minireview editor. Manuscripts should be submitted via Rapid Review.

Minireviews do not have abstracts. In the Abstract section of the submission form, put "Not applicable." The body of the Minireview may either have section headings or be set up like a Note (see above).

Guest Commentaries

Guest Commentaries are invited communications written in response to invitations issued by the editors and concern topics of interest to the broad readership of the journal that are not necessarily covered by Minireviews. They should raise issues of interest to the community of virologists, initiate or focus discussion, or propose position or consensus statements for leadership groups in research. Review of the literature, methods and other how-to papers, and responses targeted at a specific published paper are not appropriate. Guest Commentaries are subject to review.

The length may not exceed 2 printed pages, and the format is like that of a Minireview (see above). Commentaries should be submitted via Rapid Review.

Letters to the Editor

Two types of Letters to the Editor may be submitted. The first type (Comment Letter) is intended for comments on articles published previously in the journal and must cite published references to support the writer's argument. The second type (New-Data Letter) may report new, concise findings that are not appropriate for publication as full-length papers or Notes.

Letters may be **no more than 500 words long and must be typed double spaced**. Refer to a recently published Letter for correct formatting. Note that authors and affiliations are listed at the foot of the Letter. Provide only the primary affiliation for each author. Authors with the same affiliation must be listed together. The order of author names will be changed as necessary by the Jour-

nals staff to avoid repetition of an address.

All Letters to the Editor must be submitted electronically, and the type of Letter (New Data or Comment) must be selected from the drop-down list in the submission form. For Letters commenting on published articles, the cover letter should state the volume and issue in which the article was published, the title of the article, and the last name of the first author. In the Abstract section of the submission form, put "Not applicable." Letters to the Editor do not have abstracts. Both types of Letter must have a title, which must appear on the manuscript and on the submission form. Figures and tables should be kept to a minimum.

If the Letter is related to a published article, it will be sent to the editor who handled the article in question. If the editor believes that publication is warranted, he will solicit a reply from the corresponding author of the article and make a recommendation to the editor in chief. Final approval for publication rests with the editor in chief.

New-Data Letters will be assigned to an editor according to subject matter and will be reviewed by that editor and the editor in chief. Final approval for publication rests with the editor in chief.

Please note that some indexing/abstracting services do not include Letters to the Editor in their databases.

Errata

The Erratum section provides a means of correcting errors that occurred during the writing, typing, editing, or printing (e.g., a misspelling, a dropped word or line, or mislabeling in a figure) of a published article. Send Errata directly to the ASM Journals Department (1752 N St., N.W., Washington, DC 20036-2904, USA), both on disk and in hard copy (**only one hard copy is necessary**). Please see a recent issue for correct formatting.

Authors' Corrections

The Author's Correction section provides a means of correcting errors of omission (e.g., author names or citations) and errors of a scientific nature that do not alter the overall basic results or conclusions of a published article.

For omission of an author's name, the authors of the article and the author whose name was inadvertently omitted must agree, in writing, to publication of the Correction. For other issues involving authorship, including contributions and use or ownership of data and/or materials, all disputing parties must agree, in writing, to publication of the Correction. Copies of the agreement letters must accompany the Correction and be sent directly to the Journals Department. Send the Correction both on disk and in hard copy (**only one hard copy is necessary**). Please see a recent issue for correct formatting.

Corrections of a scientific nature (e.g., an incorrect unit of measurement or order of magnitude used throughout; contamination of one of numerous cultures; or misidentification of a mutant strain, causing erroneous data for only

a portion [noncritical] of the study) must be sent, both on disk and in hard copy, directly to the editor who handled the article and must be accompanied by *signed letters of agreement* from all of the authors of the article. If the editor believes that publication is warranted, he will send the Correction to the Journals Department for publication. Note that the addition of new data is not permitted.

Retractions

Retractions are reserved for major errors or breaches of ethics that, for example, may call into question the source of the data or the validity of the results and conclusions of an article. Send a Retraction and an accompanying explanatory letter *signed by all of the authors* directly to the editor in chief of the journal. The editor who handled the paper and the chairman of the ASM Publications Board will be consulted. If all parties agree to the publication and content of the Retraction, it will be sent to the Journals Department for publication.

ILLUSTRATIONS AND TABLES

Digital files that are acceptable for production (see below) must be provided for all illustrations on return of the modified manuscript. (On initial submission, the entire paper may be submitted in PDF format.)

We strongly recommend that before returning their modified manuscripts, authors check the acceptability of their digital images for production by running their files through **Rapid Inspector**, a tool provided at the following URL: <http://rapidinspector.cadmus.com/mw/>. Rapid Inspector is an easy-to-use Web-based application that takes only minutes to identify problems that may cause the file to fail at any point during the production process.

Illustrations may be continuous-tone photographs, line drawings, or composites. Color graphics may be submitted, but the cost of printing in color must be borne by the author. Suggestions about how to reduce costs and ensure accurate color reproduction are given below.

In general, digital files are not used for tables at the production stage; however, restrictions on file formats still apply (see the section on Tables below).

Since the contents of computer-generated images can be manipulated for better clarity, the Publications Board at its May 1992 meeting decreed that a description of the software/hardware used should be put in the figure legend(s).

Illustrations

File types and formats. As mentioned above, illustrations may be supplied as PDF files for reviewing purposes only on initial submission; in fact, we recommend this option to minimize file upload time. At the modification stage, production quality digital files must be submitted: TIFF or EPS files from supported applications or PowerPoint files (black and white only). Except

Macintosh		
Application	File type	
	Black and white	Color (CMYK) ^a
Adobe Illustrator 6.0, 7.0, 8.0, 9.0, and 10.0	EPS	EPS
Adobe InDesign 1.0	EPS	EPS
Adobe PageMaker 6.5	EPS	EPS
Adobe Photoshop		
4.0	TIFF	TIFF
5.0	TIFF	TIFF
5.0 LE	TIFF	N/A ^b
5.5	TIFF	TIFF
6.0	TIFF	TIFF
ChemDraw Pro 5.0	EPS/TIFF	EPS/TIFF
Corel Photo-Paint 8.0	TIFF	EPS
CorelDRAW 6.0 and 8.0	EPS/TIFF	EPS
Deneba Canvas 5.0, 6.0, 7.0, and 8.0	EPS/TIFF	EPS
Macromedia FreeHand 7.0, 8.0, and 9.0	EPS	EPS
PowerPoint '98 and 2001	PPT ^c	N/A ^b
Prism 3 by GraphPad	TIFF	N/A ^b
QuarkXpress	EPS	EPS
Synergy Kaleidagraph 3.08 and 3.51	EPS	N/A ^b

^a Color graphics must be saved and printed in the CMYK mode, not RGB.

^b ASM accepts only black-and-white, not color, graphics created with Kaleidagraph, Adobe Photoshop 5.0 LE, Prism 3 by GraphPad, and PowerPoint.

^c For instructions on saving PowerPoint files, refer to the Cadmus digital art website at <http://icjs.cadmus.com/da>.

Windows		
Application	File type	
	Black and white	Color (CMYK) ^a
Adobe Illustrator 7.0, 8.0, and 9.0	EPS	EPS
Adobe InDesign 1.0	EPS	EPS
Adobe PageMaker 6.5	EPS	EPS
Adobe Photoshop		
4.0	TIFF	TIFF
5.0	TIFF	TIFF
5.0 LE	TIFF	N/A ^b
5.5	TIFF	TIFF
6.0	TIFF	TIFF
ChemDraw Pro 5.0	EPS/TIFF	EPS/TIFF
Corel Photo-Paint 8.0 and 9.0	TIFF	EPS
CorelDRAW 7.0, 8.0, and 9.0	EPS/TIFF	EPS
Deneba Canvas 6.0 and 7.0	EPS/TIFF	EPS
Macromedia FreeHand 7.0, 8.0, and 9.0	EPS	EPS
PowerPoint '97, 2000, and XP	PPT ^c	N/A ^b
Prism 3 by GraphPad	TIFF	N/A ^b
QuarkXpress	EPS	EPS
SigmaPlot 8.01	EPS	EPS

^a Color graphics must be saved and printed in the CMYK mode, not RGB.

^b ASM accepts only black-and-white, not color, graphics created with Adobe Photoshop 5.0 LE, Prism 3 by GraphPad, and PowerPoint.

^c For instructions on saving PowerPoint files, refer to the Cadmus digital art website at <http://icjs.cadmus.com/da>.

for figures produced in PowerPoint, all graphics submitted with modified manuscripts must be bitmap, grayscale, or CMYK (*not* RGB). Acceptable file types and formats for production are given in the tables below. More-detailed instructions for preparing illustrations are available on the World Wide Web at <http://cjs.cadmus.com/da>. Please review this information before preparing your files. If you require additional information, please send an e-mail inquiry to digitalart@cadmus.com.

Minimum resolution. It is extremely important that a high enough resolution is used. Note, however, that the higher the resolution, the larger the file and the longer the upload time. Publication quality will *not* be improved by using a resolution higher than the minimum. Minimum resolutions are as follows:

- 300 dpi for grayscale and color
- 600 dpi for lettering
- 1,200 dpi for line art

Resolution requirements do not apply to graphics created in PowerPoint.

Size. All graphics **MUST** be submitted at their intended publication size; that is, the image uploaded should be 100% of its print dimensions so that no reduction or enlargement is necessary. Include only the significant portion of an illustration. White space must be cropped from the image, and excess space between panel labels and the image must be eliminated.

- Maximum width for a 1-column figure: 3½ inches (ca. 8.4 cm)
- Maximum width for a 2-column figure: 6½ inches (ca. 17.4 cm)
- Minimum width for a 2-column figure: 4¼ inches (10.8 cm)
- Maximum height: 9½ inches (25.3 cm)

Contrast. Illustrations must contain sufficient contrast to withstand the inevitable loss of contrast and detail inherent in the printing process. See also the section on color illustrations below.

Labeling and assembly. All final lettering, labeling, tooling, etc., **MUST** be incorporated into the figures. It cannot be added at a later date. If a figure number is included, it must appear well outside the boundaries of the image itself. (Numbering may need to be changed at the copyediting stage.) Each figure must be uploaded as a separate file, and any multipanel figures must be assembled into one file; i.e., rather than uploading a separate file for each panel in a figure, assemble all panels in one piece and supply them as one file.

Fonts. To avoid font problems, set all type in one of the following Type 1 PostScript fonts: Helvetica, Times Roman, European PI, Mathematical PI, or Symbol. All fonts other than these five must be converted to paths

(or outlines) in the application with which they were created. For font use in PowerPoint images, refer to the Cadmus digital art website, <http://cjs.cadmus.com/da>.

Compression. Images created with Macintosh applications may be compressed with Stuffit. Images created with Windows applications may be compressed with WINZIP.

Color illustrations. Because the process of placing ink on paper by using printing presses is different from that used to produce a photo print or a laser print and the color rendition on images viewed on a monitor depends to some extent on monitor resolution, some differences in color and contrast between the image you submit and the image printed in the journal or published online will be evident. (Figures showing red or green fluorescence and those with a significant range of colors may be difficult or impossible to reproduce exactly.) Color illustrations must be saved as either TIFF or EPS files, according to the application used (see charts above). The mode of the TIFF or EPS file must be CMYK, *not* RGB. Graphics in the RGB color space are intended for display on a monitor only and will not separate correctly for printing.

The cost of printing in color must be borne by the author. The current color costs may be accessed from the submission form in Rapid Review. Adherence to the following guidelines, in addition to the general ones above, will help to minimize costs and to ensure color reproduction that is as accurate as possible.

Include only the significant portions of illustrations so that the number of printed pages containing color figures is minimized. The individual panels of a single figure must be assembled in a single file, including any necessary labels. Optimal color reproduction will be obtained if the composites comprise panels containing similar colors of similar lightness or darkness. If necessary, make unlike panels into separate figures/files; this will increase the cost, but the color rendition will be more accurate since the two panels will be "scanned" separately.

Drawings

Submit graphs, charts, complicated chemical or mathematical formulas, diagrams, and other drawings as finished products not requiring additional artwork or typesetting. No part of the graph or drawing may be handwritten. *All* elements, including letters, numbers, and symbols, *must* be easily readable, and both axes of a graph must be labeled. Keep in mind that the journal is published both in print and online and that the same electronic files submitted by the authors are used to produce both.

When creating line art, please use the following guidelines:

1. All art **MUST** be submitted at its intended publication size. For acceptable dimensions, see the Size section above.

2. **Avoid using screens (i.e., shading)** in line art. It can be difficult and time-consuming to reproduce these images without moiré patterns. Various pattern backgrounds are preferable to screens as long as the patterns are not imported from another application. If you must use images containing screens,

- Generate the image at line screens of 85 lines per inch or lower.
 - When applying multiple shades of gray, differentiate the gray levels by at least 20%.
 - Never use levels of gray below 20% or above 70% as they will fade out or become totally black upon scanning and reduction.
3. Use thick, solid lines that are no finer than 1 point in thickness.
4. No type should be smaller than 9 point at the final publication size.
5. Avoid layering type directly over shaded or textured areas.
6. Avoid the use of reversed type (white lettering on a black background).
7. Avoid heavy letters, which tend to close up, and unusual symbols, which the printer may not be able to reproduce in the legend.
8. If colors are used, avoid using similar shades of the same color and avoid very light colors.

In figure ordinate and abscissa scales (as well as table column headings), **avoid the ambiguous use of numbers with exponents**. Usually, it is preferable to use the *Système International d'Unités* (SI) symbols (μ for 10^{-6} , m for 10^{-3} , k for 10^3 , M for 10^6 , etc.). A complete listing of SI symbols can be found in the International Union of Pure and Applied Chemistry (IUPAC) "Manual of Symbols and Terminology for Physicochemical Quantities and Units" (Pure Appl. Chem. 21:3-44, 1970). Thus, a representation of 20,000 cpm on a figure ordinate is to be made by the number 20 accompanied by the label kcpm.

When powers of 10 must be used, the journal requires that the exponent power be associated with the number shown. In representing 20,000 cells per ml, the numeral on the ordinate would be " 2×10^4 " and the label would be "10⁴ cells per ml" (not "cells per ml $\times 10^{-4}$ "). Likewise, an enzyme activity of 0.06 U/ml would be shown as 6 accompanied by the label 10^{-2} U/ml. The preferred designation would be 60 mU/ml (milliunits per milliliter).

Presentation of Nucleic Acid Sequences

Nucleic acid sequences of limited length which are the primary subject of a study may be presented freestyle in the most effective format. Longer nucleic acid sequences must be presented as figures in the following format to conserve

space. Print the sequence in lines of approximately 100 to 120 nucleotides in a nonproportional (monospace) font that is easily legible when published with a line length of 6 inches (ca. 15.2 cm). If possible, lines of nucleic acid sequence should be further subdivided into blocks of 10 or 20 nucleotides by spaces within the sequence or by marks above it. Uppercase and lowercase letters may be used to designate the exon-intron structure, transcribed regions, etc., if the lowercase letters remain legible at a 6-inch (ca. 15.2-cm) line length. Number the sequence line by line; place numerals, representing the first base of each line, to the left of the lines. **Minimize spacing between lines of sequence, leaving room only for annotation of the sequence.** Annotation may include boldface, underlining, brackets, boxes, etc. Encoded amino acid sequences may be presented, if necessary, immediately above or below the first nucleotide of each codon, by using the single-letter amino acid symbols. Comparisons of multiple nucleic acid sequences should conform as nearly as possible to the same format.

Figure Legends

Legends should provide enough information so that the figure is understandable without frequent reference to the text. However, detailed experimental methods must be described in the Materials and Methods section, not in a figure legend. A method that is unique to one of several experiments may be reported in a legend only if the discussion is very brief (one or two sentences). Define all symbols used in the figure and define all abbreviations that are not used in the text.

Tables

Tables that contain artwork, chemical structures, or shading must be submitted as illustrations in an acceptable format at the modification stage. Regular tables must be submitted either as Word, WordPerfect, or Acrobat PDF files. Note that a straight Excel file is *not* an acceptable format. Excel files must either be embedded in a Word or WordPerfect document or be converted to PDF *before* being uploaded. Although PDF files and word processing files with embedding are *not* generally acceptable for production purposes, they *are* acceptable for tables. Unlike the other parts of a manuscript, tables are not produced from the author's source files. They must be rekeyed by the printer before going into a page composition program. **If your modified manuscript contains PDF tables, select "for reviewing purposes only" at the beginning of the file upload process.**

Tables should be formatted as follows. Arrange the data so that **columns of like material read down, not across**. The headings should be sufficiently clear so that the meaning of the data is understandable without reference to the text. See the Abbreviations section (p. 15) of these Instructions for those that should be used in tables. Explanatory footnotes are acceptable, but more extensive table "legends" are not. Footnotes should not include detailed descriptions of the experiment. Tables

must include enough information to warrant table format; those with fewer than six pieces of data will be incorporated into the text by the copy editor. Table 1 is an example of a well-constructed table.

TABLE 1. Distribution of protein and ATPase in fractions of dialyzed membranes*

Membrane	Fraction	ATPase	
		U/mg of protein	Total U
Control	Depleted membrane	0.036	2.3
	Concentrated supernatant	0.134	4.82
EI treated	Depleted membrane	0.034	1.98
	Concentrated supernatant	0.11	4.6

* Specific activities of ATPase of nondepleted membranes from control and treated bacteria were 0.21 and 0.20, respectively.

Cover Photographs and Drawings

JVI publishes photographs and drawings on the front cover. Invitations are issued to authors whose manuscripts are returned for modification or whose manuscripts have been accepted for publication in JVI; material should be related to the work presented in the manuscript. Unsolicited material will also be considered, however. No material submitted for consideration will be returned to the author. Copyright for the chosen material must be transferred to ASM. A short description of the cover material will be included at the end of the table of contents or the author index of the issue. Technical specifications are available from the cover editor, Daniel DiMaio (e-mail: daniel.dimaio@yale.edu).

NOMENCLATURE

Chemical and Biochemical Nomenclature

The recognized authority for the names of chemical compounds is *Chemical Abstracts* (Chemical Abstracts Service, Ohio State University, Columbus) and its indexes. *The Merck Index*, 13th ed. (Merck & Co., Inc., Whitehouse Station, N.J., 2001), is also an excellent source. For biochemical terminology, including abbreviations and symbols, consult *Biochemical Nomenclature and Related Documents* (1978; reprinted for The Biochemical Society, London, England) and the instructions to authors of the *Journal of Biological Chemistry* and the *Archives of Biochemistry and Biophysics* (first issues of each year).

Do not express molecular weight in daltons; molecular weight is a unitless ratio. Molecular mass is expressed in daltons.

For enzymes, use the recommended (trivial) name assigned by the Nomenclature Committee of the International Union of Biochemistry (IUB) as described in *Enzyme Nomenclature* (Academic Press, Inc., New York, N.Y., 1992). If a nonrecommended name is used, place

the proper (trivial) name in parentheses at first use in the abstract and text. Use the EC number when one has been assigned, and express enzyme activity either in katal (preferred) or in the older system of micromoles per minute.

For nomenclature of restriction enzymes, DNA methyltransferases, homing endonucleases, and their genes, refer to the article by Roberts et al. (*Nucleic Acids Res.* 31:1805–1812, 2003).

Nomenclature of Mice

For mouse strain and genetic nomenclature, ASM encourages authors to refer to the guidelines set forth by the International Committee on Standardized Genetic Nomenclature for Mice, available on the Mouse Genome Database home page at <http://www.informatics.jax.org> and in *Genetic Variants and Strains of the Laboratory Mouse*, 3rd ed. (M. F. Lyon et al., ed., Oxford University Press, Oxford, England, 1996).

Nomenclature of Viruses

Names used for viruses should be those approved by the International Committee on Taxonomy of Viruses (ICTV) and published in *Virus Taxonomy: Classification and Nomenclature of Viruses, Seventh Report of the International Committee on Taxonomy of Viruses* (M. H. V. van Regenmortel et al., ed., Academic Press, San Diego, Calif., 2000). In addition, the recommendations of the ICTV regarding the use of species names should generally be followed: when the entire species is discussed as a taxonomic entity, the species name, like other taxa, is italic and has the first letter and any proper nouns capitalized (e.g., *Tobacco mosaic virus*, *Murray Valley encephalitis virus*). When the behavior or manipulation of individual viruses is discussed, the vernacular (e.g., tobacco mosaic virus, Murray Valley encephalitis virus) should be used. If desired, synonyms may be added parenthetically when the name is first mentioned. Approved generic (or group) and family names may also be used.

Nomenclature of Bacteria

Binary names, consisting of a generic name and a specific epithet (e.g., *Escherichia coli*), should be used for all bacteria. Names of categories at or above the genus level may be used alone, but specific and subspecific epithets may not. A specific epithet must be preceded by a generic name, written out in full the first time it is used in a paper. Thereafter, the generic name should be abbreviated to the initial capital letter (e.g., *E. coli*), provided there can be no confusion with other genera used in the paper. Names of all taxa (kingdoms, phyla, classes, orders, families, genera, species, and subspecies) are printed in italics; strain designations and numbers are not.

Genetic Nomenclature

To facilitate accurate communication, it is important that standard genetic nomenclature be used whenever possible and that deviations or proposals for new naming systems be endorsed by an appropriate authoritative body. Review and/or publication of submitted manuscripts that contain new or nonstandard nomenclature may be delayed by the editor or the Journals Department so that they may be reviewed by the Genetics and Genomics Committee of the ASM Publications Board.

Before submission of manuscripts, authors may direct questions on genetic nomenclature to the committee's chairman: Maria Costanzo (e-mail: maria@genome.stanford.edu). Such a consultation should be mentioned in the manuscript submission letter.

When appropriate for viral genetic systems, use the recommendations of Demerec et al. (Genetics 54:61–76, 1966) as a guide.

(i) Phenotype designations must be employed when mutant loci have not been identified or mapped. They can also be used to identify the protein product of a gene, e.g., the OmpA protein. Phenotype designations generally consist of three-letter symbols; these are *not* italicized and the first letter of the symbol is capitalized. It is preferable to use Roman or Arabic numerals (instead of letters) to identify a series of related phenotypes. Thus, a series of bacteriocin-tolerant mutants might be designated TolI, TolII, TolIII, etc., or a series of nucleic acid polymerase mutants might be designated Pol1, Pol2, Pol3, etc. Wild-type characteristics can be designated Tol⁺ or Pol⁺, and, when necessary for clarity, negative superscripts (Tol[−] Pol[−]) can be used to designate mutant characteristics. Lowercase superscript letters may be used to further delineate phenotypes (e.g., Str^r for streptomycin resistance). Phenotype designations should be defined.

(ii) Genotype designations are also indicated by three-letter locus symbols. These are lowercase italic (e.g., *pol* *src*). If several loci govern related functions, these are distinguished by italicized capital letters following the locus symbol.

(iii) Wild-type alleles are indicated with a superscript plus (*ara*⁺ *his*⁺). A superscript minus is not used to indicate a mutant locus; thus, one refers to an *ara* mutant rather than an *ara*[−] strain.

(iv) The rules for genetic nomenclature of viruses (phages) differ from those of bacteria. As a general rule, the entire description of a virus is italicized, including the designations *am* or *sus* (amber suppressible) and *ts* (temperature sensitive). Superscripts are employed to indicate hybrid genomes. Genetic symbols may be one, two, or three letters. For example, a mutant strain of λ might be designated λ c1857 *int2* *red114* *sus411*; this strain carries mutations in genes *cl*, *int*, and *red* and a suppressible (*sus*) mutation in gene *A*. A strain designated λ *imm*²¹ *att*⁴³⁴ would represent a hybrid of phage λ which carries the immunity (*imm*) region of phage 21 and the attachment (*att*) region of phage 434. Host DNA insertions into viruses should be delineated by square

brackets, and the genetic symbols and designations for such inserted DNA should conform to those employed for the host genome. Genetic symbols for phage λ can be found in reports by Echols and Murialdo (Microbiol. Rev. 42:577–591, 1978) and Szybalski and Szybalski (Gene 7:217–270, 1979).

“Mutant” versus “mutation.” Authors are reminded of the distinction between a *mutation* (an alteration of the primary sequence of the genetic material) and a *mutant* (a strain carrying one or more mutations). One may speak about the mapping of a mutation, but one cannot map a mutant. Likewise, a mutant has no genetic locus, only a phenotype.

“Homology” versus “similarity.” For use of terms that describe relationships between genes, consult the articles by Theissen (Nature 415:741, 2002) and Fitch (Trends Genet. 16:227–231, 2000).

“Homology” implies a relationship between genes that share a common evolutionary origin; partial homology is not recognized. When sequence comparisons are discussed, it is more appropriate to use the term “percent sequence similarity” or “percent sequence identity,” as appropriate.

ABBREVIATIONS AND CONVENTIONS

Verb Tense

ASM strongly recommends that for clarity you use the past tense to narrate particular events in the past, including the procedures, observations, and data of the study that you are reporting. Use the present tense for your own general conclusions, the conclusions of previous researchers, and generally accepted facts. Thus, most of the abstract, Materials and Methods, and Results will be in the past tense, and most of the introduction and some of the Discussion will be in the present tense.

Be aware that it may be necessary to vary the tense in a single sentence. For example, it is correct to say “White (30) demonstrated that XYZ cells grow at pH 6.8,” “Figure 2 shows that ABC cells failed to grow at room temperature,” and “Air was removed from the chamber and the mice died, which proves that mice require air.” In reporting statistics and calculations, it is correct to say “The values for the ABC cells are statistically significant, indicating that the drug inhibited . . .”

For an in-depth discussion of tense in scientific writing, see p. 207–209 in *How To Write and Publish a Scientific Paper*, 5th ed.

Abbreviations

General. Abbreviations should be used as an aid to the reader, rather than as a convenience to the author, and therefore their use should be limited. Abbreviations other than those recommended by the IUPAC-IUB (Bio-

chemical Nomenclature and Related Documents, 1978) should be used only when a case can be made for necessity, such as in tables and figures.

It is often possible to use pronouns or to paraphrase a long word after its first use (e.g., "the drug" or "the substrate"). Standard chemical symbols and trivial names or their symbols (folate, Ala, Leu, etc.) may also be used.

It is strongly recommended that all abbreviations except those listed below be introduced in the first paragraph in Materials and Methods. Alternatively, define each abbreviation and introduce it in parentheses the first time it is used; e.g., "cultures were grown in Eagle minimal essential medium (MEM)." Generally, eliminate abbreviations that are not used at least three times in the text (including tables and figure legends).

Not requiring introduction. In addition to abbreviations for *Système International d'Unités* (SI) units of measurement, other common units (e.g., bp, kb, and Da), and chemical symbols for the elements, the following should be used without definition in the title, abstract, text, figure legends, and tables: DNA (deoxyribonucleic acid); cDNA (complementary DNA); RNA (ribonucleic acid); cRNA (complementary RNA); RNase (ribonuclease); DNase (deoxyribonuclease); rRNA (ribosomal RNA); mRNA (messenger RNA); tRNA (transfer RNA); AMP, ADP, ATP, dAMP, dATP, GTP, etc. (for the respective 5' phosphates of adenosine and other nucleosides) (add 2', 3', or 5' when needed for contrast); ATPase, dGTPase, etc. (adenosine triphosphatase, deoxyguanosine triphosphatase, etc.); NAD (nicotinamide adenine dinucleotide); NAD⁺ (nicotinamide adenine dinucleotide, oxidized); NADH (nicotinamide adenine dinucleotide, reduced); NADP (nicotinamide adenine dinucleotide phosphate); NADPH (nicotinamide adenine dinucleotide phosphate, reduced); NADP⁺ (nicotinamide adenine dinucleotide phosphate, oxidized); poly(A), poly(dT), etc. (polyadenylic acid, polydeoxythymidylic acid, etc.); oligo(dT), etc. (oligodeoxythymidylic acid, etc.); UV (ultraviolet); PFU (plaque-forming units); CFU (colony-forming units); MIC (minimal inhibitory concentration); Tris [tris(hydroxymethyl)amino-methane]; DEAE (diethylaminoethyl); EDTA (ethylenediaminetetraacetic acid); EGTA [ethylene glycol-bis (β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid]; HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid); PCR (polymerase chain reaction); and AIDS (acquired immunodeficiency syndrome). Abbreviations for cell lines (e.g., HeLa) also need not be defined.

The following abbreviations should be used without definition in tables:

amt (amount)	SE (standard error)
approx (approximately)	SEM (standard error of the mean)
avg (average)	
concn (concentration)	sp act (specific activity)
diam (diameter)	sp gr (specific gravity)
expt (experiment)	temp (temperature)

expt (experimental)	tr (trace)
ht (height)	vol (volume)
mo (month)	vs (versus)
mol wt (molecular weight)	wk (week)
no. (number)	wt (weight)
prepn (preparation)	yr (year)
SD (standard deviation)	

Reporting Numerical Data

Standard metric units are used for reporting length, weight, and volume. For these units and for molarity, use the prefixes m, μ , n, and p for 10^{-3} , 10^{-6} , 10^{-9} , and 10^{-12} , respectively. Likewise, use the prefixes c for 10^{-2} and k for 10^3 . Avoid compound prefixes such as $\mu\mu$ or $\mu\mu\mu$. Use $\mu\text{g/ml}$ or $\mu\text{g/g}$ in place of the ambiguous ppm. Units of temperature are presented as follows: 37°C or 324 K.

When fractions are used to express units such as enzymatic activities, it is preferable to use whole units, such as g or min, in the denominator instead of fractional or multiple units, such as μg or 10 min. For example, "pmol/min" is preferable to "nmol/10 min," and " $\mu\text{mol/g}$ " is preferable to "nmol/ μg ." It is also preferable that an unambiguous form such as exponential notation be used; for example, " $\mu\text{mol g}^{-1} \text{min}^{-1}$ " is preferable to " $\mu\text{mol/g/min}$." Always report numerical data in the appropriate SI units.

For a review of some common errors associated with statistical analyses and reports, plus guidelines on how to avoid them, see the article by C. Olsen (*Infect. Immun.* 71:6689-6692, 2003).

Isotopically Labeled Compounds

For simple molecules, labeling is indicated in the chemical formula (e.g., $^{14}\text{CO}_2$, $^3\text{H}_2\text{O}$, and $\text{H}_2^{35}\text{SO}_4$). Brackets are not used when the isotopic symbol is attached to the name of a compound that in its natural state does not contain the element (e.g., ^{32}S -ATP) or to a word that is not a specific chemical name (e.g., ^{131}I -labeled protein, ^{14}C -amino acids, and ^3H -ligands).

For specific chemicals, the symbol for the isotope introduced is placed in square brackets directly preceding the part of the name that describes the labeled entity. Note that configuration symbols and modifiers precede the isotopic symbol. The following examples illustrate correct usage:

[^{14}C]urea	L-[methyl- ^{14}C]methionine
[2,3- ^3H]serine	[α - ^{14}C]lysine
[γ - ^{32}P]ATP	UDP[U- ^{14}C]glucose
SV40 [^{32}P]DNA	fructose 1,6-[1- ^{32}P]bisphosphate

JVI follows the same conventions for isotopic labeling as the *Journal of Biological Chemistry*, and more-detailed information can be found in the instructions to authors of that journal (first issue of each year).

“Of mice and men”: values and liabilities of the athymic nude mouse model in anticancer drug development

L.R. Kelland*

Antisoma Research Laboratories, St Georges Hospital Medical School, Cranmer Terrace, London SW17 0QS, UK

Received 22 August 2003; accepted 16 November 2003

Abstract

Human tumour xenografts implanted subcutaneously (s.c.) into immunosuppressed mice have played a significant role in pre-clinical anticancer drug development for the past 25 years. Their use as a predictive indicator of probable clinical activity has been validated for cytotoxics. A retrospective analysis for 39 compounds where both extensive xenograft testing and Phase II clinical data were available, performed by the National Cancer Institute (NCI), has shown that 15/33 agents (45%) with activity in more than one-third of xenografts showed clinical activity ($P=0.04$). However, with the exception of non-small cell lung cancer, activity within a particular histological type of the xenograft generally did not predict for clinical activity in the same tumour. Today, the question (largely unanswered) is how useful is the xenograft model (particularly the traditional s.c. model) in contemporary cancer drug discovery? There are many variables when conducting xenograft experiments which impact on outcome; viz, site of implantation, growth properties of the xenograft and size when treatment is initiated, agent formulation, scheduling, route of administration and dose and the selected endpoint for assessing activity. The xenograft model remains of value in current preclinical cancer drug development, especially when such studies give due consideration to the above variables and are based on sound mechanistic (e.g. status of the selected target in the chosen model) and pharmacological (e.g. use of formulated agent) principles. Dependent upon the drug target, a slowing of xenograft tumour growth (cytostatic effect) rather than tumour shrinkage might be the major observed effect. Human tumour xenografts are also particularly useful in determining pharmacodynamic markers of response for subsequent clinical application. Nevertheless, it needs to be kept in mind that the use of xenografts is relatively time-consuming and expensive, raises animal ethical issues and there are instances where the model is inappropriate as a likely predictor of clinical outcome (e.g. inhibitors of the metastatic process and anti-angiogenic strategies as the vasculature is of murine origin).

© 2004 Elsevier Ltd. All rights reserved.

Keywords: Athymic nude mouse model; Anti-cancer drug development; Review; Human tumour xenografts; Limitations

1. Introduction

Contemporary anticancer drug development is a multi-million dollar and time-consuming business. Typically, from concept to the completion of Phase III clinical trials and gaining regulatory approval requires in excess of 10 years and as much as 500 million dollars. For the first quarter of a century of modern cancer drug development (circa 1945–1969), thousands of generally randomly produced molecules were tested in mice bearing rapidly growing murine leukaemias (e.g. P388 and L1210) [1]. In 1969, came the first report of the growth of a human tumour in an immunodeficient “nude”

(athymic) mouse [2]. Since then, human tumour xenografts grown in nude [3] or in mice with severe combined immunodeficiency (SCID) [4] have covered all of the major tumour types and represented the mainstay of preclinical anticancer drug development testing *in vivo*.

The modern paradigm for anticancer drug discovery, as widely used by drug companies and within some academic groups, comprises a series of carefully constructed steps that are designed to rapidly and efficiently allow “proof of principle”, pharmaceutically-tractable, molecules to be tested in Phase I and II clinical trials. Such a cascade (Fig. 1) may be envisaged as a large number of molecules feeding into a series of iterative stop/go tests of increasing biological complexity. The concept of “therapeutic index”, that is the demonstration of antitumour efficacy at doses well below those

* Tel.: +44-208-772-4393; fax: +44-208-767-1809.
E-mail address: lloyd@antisoma.com (L.R. Kelland).

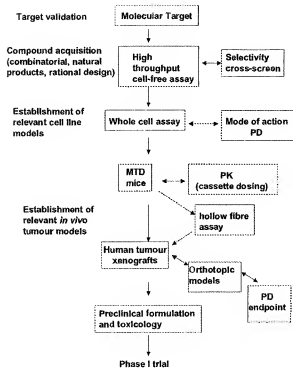


Fig. 1. Generic contemporary drug evaluation cascade. PD, pharmacodynamics; PK, pharmacokinetics; MTD, maximum tolerated dose.

causing severe toxicities, is also a long-established paradigm in preclinical drug discovery [5]. A key part of the preclinical stage of the process, and often representing a significant bottleneck, is the demonstration of antitumour efficacy in the “relevant” tumour model *in vivo*.

However, what is the place for the nude mouse xenograft model in cancer drug development today in this post-genomic era? What are its values? What are its limitations? Is testing *in vivo* even required—could predictive answers be obtained solely using human tumour cells in culture or even using *in silico* methodology, thus avoiding the increasing ethical issues raised by animal experimentation? As opposed to the previous era of “cytotoxic” cancer drugs, contemporary cancer drug development encompasses a wide variety of approaches generally based on attacking specific molecular targets where often, cytostatic rather than cytotoxic effects may be predicted. Thus, a slowing of tumour growth rather than shrinkage may occur. This may require a re-evaluation of the *in vivo* models developed and validated using cytotoxic drugs when testing such agents. Even within mouse models, is it the case that, in some instances, murine syngeneic models (often dismissed since the advent of human tumour models), transgenic models or orthotopic models may be more appropriate to use than human subcutaneously (s.c.) implanted xenografts in immune-suppressed animals? In some cases, maybe there is no appropriate preclinical model?

In an industry where time is paramount, should we dispense with relatively slow and laborious xenograft efficacy determinations in favour of some more rapid, higher-throughput alternative (e.g. the hollow fibre assay [6])?

Hence, this article aims to provide a critical review of the role of human tumour xenografts transplanted in athymic (or SCID) mice in contemporary cancer drug development. Both values and liabilities will be discussed specifically in the context of experience with firstly cytotoxic platinum-based molecules and secondly with small molecules targeted against contemporary cancer targets (e.g. farnesyltransferase, heat shock protein 90, telomerase and tumour vasculature).

2. Human tumour xenografts: a validated model in the development of cytotoxic drugs

Within a few years of the original description of the nude mouse model, the Developmental Therapeutics Program at the National Cancer Institute (NCI) adopted, in 1976, the use of 3 human tumour s.c. xenografts (one representing each of colon, CX-1, breast, MX-1, and lung, LX-1, cancers) into its *in vivo* cancer drug screening programme [7]. Since then, although far greater emphasis has been placed on initial screening using panels of disease-oriented human tumour cell lines [8], the xenograft model has remained important. Early studies by Giovanella and colleagues [3] indicated that higher take rates were obtainable using cultured cells rather than when tumours were transplanted directly from patient biopsies (but see below). Later efforts at the NCI focused on the establishment of s.c. xenograft models from each of the 60 human tumour cell lines used in the *in vitro* screen [7].

In parallel, from the outset of xenografts being established from a variety of tumour types, numerous studies during the 1970s evaluated the predictive utility of the xenograft model in terms of responsiveness to “standard” anticancer drugs in relation to their effectiveness against corresponding tumour types in patients. Steel and colleagues established s.c. xenografts from a variety of tumour types (actually in conventional CBA mice that had been immuno-suppressed by thymectomy, cytosine arabinoside treatment and whole-body irradiation) and recorded that “human tumour xenografts broadly maintain the level of chemotherapeutic responsiveness of the source tumours in man” [9]. For example, xenografts derived from testicular teratomas and small cell lung tumours (both of which are relatively responsive to drugs in man) responded well, whereas xenografts derived from melanomas, colorectal and non-small cell lung tumours responded poorly. Overall, a total of 329 tumours were investigated; 34 (52% success rate) of colorectal xenografts were established, 9

(60%) of melanomas, 11 (23%) of ovarian cancers, 8 (10%) of breast cancers and 14 (30%) of testicular tumours. In addition, it was found that the histological characteristics of the source tumours were generally well maintained by xenografts. Responses to a variety of "standard" drugs available at this time were assessed either *in situ* (by growth delay from calliper measurements) or *ex vivo* by assessment of clonogenic cell survival using a soft-agar based assay [10].

From the early days, human tumour xenografts were established either by direct implantation of patient biopsy material or via inoculation of continuous human tumour cell lines. A particularly important large panel of xenografts, derived directly from biopsies, has been established by Fiebig and colleagues at the University of Freiburg in Germany [11]. More than 1600 tumours have been transplanted *s.c.* into nude mice and more than 300 xenografts established, representative of all of the major tumour types. A comparison of drug response in the xenograft compared with that in the patient was made in 80 cases in 55 xenografts using either an *in vivo* assay (a comparison of treated versus control tumour volumes) or *ex vivo* using a soft agar clonogenic assay from disaggregated tumours. In accordance with the earlier studies of Steel and colleagues alluded to above, the xenografts predicted correctly for clinical response in 19/21 (90%) of occasions when using the *in vivo* assay (this was reduced a little to 60% using the clonogenic assay) and predicted for resistance in 57/59 (97%) of occasions when using the *in vivo* assay (92% for the clonogenic assay).

In addition, the response pattern of more recently discovered clinically active drugs, paclitaxel, gemcitabine, docetaxel, vindesine and topotecan, was determined in 187 xenografts. Overall, the 5 drugs induced remissions in 24% (45/187) of the xenografts studied, whereas minor regressions or no change occurred in 13% of cases while 63% (117/187) of xenografts progressed on treatment. These findings are similar to the overall response rates recorded for monotherapy clinical trials with these agents. In addition, more responses (37%) were seen in a sub-group of tumours classified by the authors as clinically sensitive (small cell and non-small cell lung, breast, head and neck, leukaemia, melanoma, non-Hodgkin's lymphoma, gastric, testis) in comparison with those designated as resistant (4%; bladder, colon, cervix, central nervous system (CNS), hepatoma, mesothelioma, ovary, pancreas, prostate, renal soft tissue sarcoma). In recent years, this panel has been used to identify new drugs.

3. Use in cytotoxic cancer drug development

In addition, during the 1980s, many groups established disease-specific panels of xenografts from patient

biopsies for the purposes of either studying tumour biology, responses to existing therapy (radiotherapy or chemotherapy) or for the discovery of specific classes of new drugs. A particular emphasis at the Institute of Cancer Research, London, was to establish parallel panels of *in vitro* cell lines (as a renewable source for biochemical and molecular studies) and corresponding *in vivo* xenograft counterparts (for pharmacological studies). This was firstly achieved for carcinoma of the cervix where 9 serially transplantable xenograft lines (and 4 parallel continuous cell lines) were established from 23 original biopsies [12,13]. The *in vivo* response to 3 commonly used drugs at the time (cisplatin, etoposide and bleomycin) performed in 3 xenografts showed the models to be relatively unresponsive [13]. A comparison of the *in vitro* and *in vivo* radiation response for 3 of the cell lines/xenografts revealed a general tendency for the *in vivo* results to follow that predicted from the *in vitro* studies [14].

The approach of using disease-specific *in vitro* cell lines and corresponding xenografts for cancer drug discovery was used in a programme designed to discover more effective analogues of cisplatin and carboplatin. To begin with, a panel of 23 serially transplantable ovarian cancer xenografts were established in female nude mice from 42 donor samples (from both previously treated and untreated patients) [15]. Initial "calibration" studies determined the response of 16 of the xenografts to cisplatin and carboplatin and two other platinum-containing agents (iproplatin and tetraplatin which were undergoing clinical testing in the late 1980s). Three distinct patterns of response were observed; comparative responsiveness to all 4 platinum drugs (2 lines), resistance to all 4 drugs (5 lines) and individual drug-specific responses (9 lines). Notably, it was also possible to confirm that in 8/9 lines, the therapeutic response of the xenograft reflected that recorded in the corresponding ovarian cancer patient receiving platinum-based chemotherapy.

Secondly, attempts were made to establish parallel cell line and xenograft counterparts; a total of 8 such paired models were established and sensitivities to cisplatin and carboplatin compared *in vitro* versus *in vivo* [16]. This study highlighted the comparative ease of establishing xenografts from cell lines (6/8 paired models were derived in this way) versus the comparative difficulty of establishing cell lines from xenografts. Although this was attempted on numerous occasions over several years for all 16 ovarian models used in the above described platinum calibration studies, cell lines were only derived from two xenografts (HX62 and PXN94). This was predominantly because of the propensity for primary cultures derived from xenografts to be rapidly overrun by murine fibroblasts. By contrast, as described above for cervical carcinoma, a greater success rate was obtained by splitting an original biopsy sample, part for

cell line establishment and part for direct s.c. implantation into nude mice. Notably, despite the presumed very different selection pressures operating with cell line versus xenograft establishment, a high statistically significant positive correlation for *in vitro* sensitivity versus *in vivo* responsiveness was obtained for both cisplatin and carboplatin [16]. This is illustrated in Fig. 2 and led directly to the incorporation of these paired models into a platinum drug discovery evaluation cascade [17].

The ovarian cancer models were further extended in the platinum drug discovery context by the establishment of pairs of platinum-drug sensitive and resistant lines [18]. In contrast to most drug resistance studies, where resistance was generated through repeated exposure of tumour cell lines to increasing concentrations of drug (often in excess of those achievable in plasma *in vivo*), 7/8 of these platinum-resistant ovarian cancer models were established *in vivo* by repeated treatment of mice bearing xenografts (and repeated passaging into new donors) to cisplatin, carboplatin, iproplatin or tetraplatin. Biochemical studies elucidating the major mechanisms of platinum drug resistance in these acquired resistant cell line (and xenograft) models were then carried out. Paired cisplatin sensitive and acquired or intrinsic resistant cell line and xenograft models were then used over several years to screen in excess of 500 platinum analogues, with the aim of finding new platinum drugs possessing a greater spectrum of activity than cisplatin/carboplatin. Two drugs, the orally bioavailable JM216 (Satraplatin) and AMD473 were introduced into the clinic [19]. While both have demonstrated clinical activity (e.g. in ovarian and hormone-refractory prostate cancer) the activity of AMD473 against acquired cisplatin-resistant ovarian cancer was relatively modest [20]. In many respects, this was reflected in the preclinical xenograft data for this compound as pronounced activity against intrinsic or acquired cisplatin resistant ovarian carcinoma xenografts was limited to one model (CH1cisR) [21].

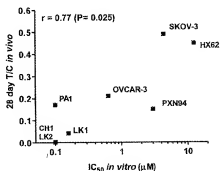


Fig. 2. Positive correlation for cisplatin between *in vivo* response of xenografts versus *in vitro* potency for corresponding cell line, across 8 human ovarian carcinoma models (replotted from Ref. 16). IC₅₀, concentration causing 50% cell growth inhibition; T/C, treated versus control values.

The most compelling evidence for the value of the s.c. xenograft model in cytotoxic cancer drug development comes from an extensive retrospective analysis from the NCI [22]. For 39 compounds, where both xenograft and Phase II data were available, activity (defined as either an increase of mouse survival by more than 25% or by a reduction in tumour weight in treated versus controls to greater than 40%) in at least one-third of tested xenografts, predicted for activity in at least some Phase II trials. Clinical activity (defined as responses in at least two tumour types) was not observed for any of the 6 agents where preclinical activity was seen in fewer than one-third of xenograft models tested. By contrast, 15/33 agents (45%) with activity in more than one-third of xenografts showed clinical activity ($P=0.04$). Of the 39 agents evaluated, many may be considered as "standard" anticancer agents in use today (such as methotrexate, chlorambucil, 5-fluorouracil, cisplatin, paclitaxel, vinblastine, irinotecan, docetaxel, doxorubicin and cyclophosphamide). These data suggest that, at least for existing cytotoxic anticancer drugs, the s.c. xenograft model is of predictive value. However, activity within a particular histological type of xenograft generally did not predict for clinical activity in the same tumour, with the exception of non-small cell lung cancer.

Interestingly, the NCI analysis also investigated whether correlations exist between activity in xenografts versus activity in a shorter-term (6-day) *in vivo* assay (the hollow fibre assay) or even potency against cell lines *in vitro*. Positive correlations could help in the preselection of compounds for xenograft testing and thereby reduce the number of animals used. The hollow fibre assay is a hybrid *in vitro/in vivo* assay whereby particular cell lines from the 60 cell line panel may be grown and compounds rapidly tested *in vivo* [23,6]. In this assay, tumour cells (normally two of each of breast, ovarian, glioma, colon, melanoma and non-small cell lung) are sealed into polyvinylidene fluoride (PVDF) biocompatible hollow fibres and implanted into mice in two anatomically separate sites, intraperitoneally (i.p.) and s.c., and mice dosed i.p. with the test compound at two dose levels for up to 4 days. Two days later, fibres are removed and the numbers of remaining viable cells compared in treated versus control groups [Ref. Decker S, Hollingshead M, Bonomi C, Carter J, Sausville E (2004). The hollow fibre model in cancer during screening: the NCI experience. *Eur J Cancer*, 40, this issue.

Interestingly, increased activity in the hollow fibre assay (especially with i.p. implanted fibres) also predicted for increased xenograft activity. Overall, 35% of 537 compounds tested had activity in at least one xenograft, whereas this increased to 63% for compounds exhibiting a response in more than 10 i.p. fibres [22]. The correlation did not occur with s.c. implanted fibres; this may be because there is insufficient time for angio-

genesis to the fibres to occur in the s.c. setting, thereby potentially limiting drug delivery to the tumour cells [24]. There was also a strong correlation between potency in the 60 cell-line screen and activity in the hollow fibre assay. 56% of compounds with a mean 50% growth inhibition of below 0.032 μM were active in at least six i.p. fibres, whereas only 4% of compounds with a potency of 100 μM were active to the same level.

Overall, taking all of the above into consideration, one may reasonably conclude that, at least for cytotoxic cancer drugs, the human tumour xenograft model, is a good predictor of clinical activity. Therefore, it seems reasonable and valuable, to continue the testing of new cytotoxics using xenografts. This is especially the case when used in combination with sound pharmaceutical and pharmacological principles (see below). Notably, some regulatory authorities (e.g. the European Medicines Evaluation Agency (EMEA) in its guidance notes on the preclinical evaluation of anticancer medicinal products, encourage the use of xenograft studies (<http://www.eudra.org/emea.html>).

4. A standard operating procedure (SOP) for conducting xenograft experiments? 99 ways to determine efficacy in a xenograft model: values and limitations

Before considering whether xenografts should still be used in contemporary cancer drug discovery (see below), it is critical to appreciate and understand each of the many variables that exist in the use of xenografts in drug testing. The cancer drug discovery literature is overwhelmed with preclinical studies invariably describing “active” new molecules where it is then proposed that these should be tested in the clinic. Commonly, such studies have involved human tumour xenografts, but used in a multitude of ways to assess the activity of molecules, regrettably, often with limited scientific rationale in terms of predictive value and integrating how the corresponding drugs will most likely be used in cancer patients. Hence, one needs to interpret xenograft studies with caution and bearing in mind the

many variables that exist. Major variables are listed in Table 1 and include the origin of the tumour (i.e. cell line versus patient biopsy), target/receptor status of the tumour, the site of tumour implantation (e.g. s.c., i.p., orthotopic), the size of tumour at the onset of agent treatment, growth rate and growth characteristics, agent dose, formulation, scheduling and route of administration, and experimental endpoints. In the worst case scenario, many mice have been used in screening experiments of questionable pharmacological relevance and predictive value where poorly formulated agents (e.g. in Tween-based suspensions) have been administered i.p. to mice within 1–2 days of these mice being inoculated, also i.p., with suspensions of tumour cells and early assessments of “activity” made. Such studies add little to *in vitro* findings. Furthermore, to date, xenografts have often been used without characterisation at the molecular level for the particular target being addressed. This has made analyses of the value of the xenograft model in predicting for activity in patients, problematic, and has resulted in a wide-range of views being expressed. In view of the above-described large retrospective analysis by the NCI, proposals such as “activity was seen in the HT29 colon xenograft, therefore clinical trials should be conducted in patients with colon cancer” are naïve, especially in the absence of any consideration of pharmacological and pharmacodynamic principles. Let us consider some of these important variables.

4.1. Origin of the xenograft model

In general, xenografts derived directly from patient biopsies, in contrast to those derived from continuous cell lines, appear to retain better the morphological and molecular marker properties reminiscent of the source tumours in man. By contrast, xenografts derived from cell lines generally show a more homogeneous, undifferentiated histology (and, on occasion, loss of the target receptors/proteins), probably indicative of the higher selection pressure *in vitro* during extensive culturing. However, a disadvantage with lines established

Table 1
Xenograft testing: variables

Variable	Comment
Origin of tumour	Cell line or patient biopsy
Site of tumour implantation	S.c., i.p., orthotopic
Growth properties of tumour	Doubling time, degree of necrosis, stromal compartment
Size of tumour at onset of treatment	‘chemoprevention’, ‘early’, ‘advanced’ stage
Target status of tumour	Receptor/antigen density, presence of target
Agent formulation	Suspension or formulated, correlation with proposed clinical formulation?
Agent scheduling	Bolus, chronic, route of administration (i.v., i.p., intratumoral, oral)
Agent dose	Maximum tolerated? Pharmacokinetic, Pharmacodynamic correlation?
Endpoints	Increase in life-span. T/C, growth delay. When assessed.

s.c., subcutaneously; i.p., intraperitoneally; i.v., intravenously. T/C treated versus control values.

directly from biopsies is the difficulty in establishing cell lines from xenografts (as described above for ovarian cancer). Therefore, it is relatively difficult to establish parallel *in vitro* cell line (valuable as a continued source of pure human tumour cells for biochemical and molecular studies) and corresponding xenograft lines (valuable for pharmacological and pharmacodynamic studies). However, overall it appears that both xenografts derived from cell lines (e.g. the NCI studies) and those derived directly from patient biopsies (e.g. the Frieburg panel) provide some predictive power for selecting cytotoxics with clinical activity.

4.2. Site of xenograft implantation

The site of tumour implantation is another important variable. While most xenograft experiments use s.c. implantation (where it is then relatively easy to assess antitumour effects through the use of callipers to determine tumour diameter/volume), there are many reports of differences in biological behaviour (e.g. ability to metastasise and receptor/target status) when tumours are grown s.c. relative to orthotopically [25]. In addition, one needs to bear in mind the pharmacological limitation (relative to chemotherapy in man) of administering the test compound at the same site as the tumour (e.g. i.p. or intratumorally).

4.3. Growth characteristics of the model

It is also widely recognised that not all xenograft models are usable for compound screening because of their growth properties. Sometimes growth is too slow (over many months>this is often an issue with transgenic models as well), or too inconsistent/erratic/non-linear with time, or they possess cystic or necrotic areas, even at relatively small tumour volumes. The occurrence of cystic or diffuse necrotic areas (even to the edges of some tumours) can often comprise 20–80% of the tumour mass, depending upon the model and number of passages in mice. Although this effect may be less of a problem when SCID mice are used as hosts relative to athymic nude mice [26], the issue does require consideration when using new models. For example, from 1600 tumour biopsy samples originally implanted into nude mice by the Frieburg group, 300 xenograft lines were established and 60 of these extensively studied [11]. Second, of 9 cervical carcinoma xenograft lines established, only 3 were applicable to routine use because of either particularly slow growth and/or the tendency for possessing diffuse necrosis [13].

4.4. Stage at which the treatment begins

The activity of a test molecule can also be critically dependent upon the stage (size) of the tumour at the

onset of treatment. In some instances, (which may be relevant to how this class of agent may be applied to man) agents are administered at the same time as tumour implantation (“chemoprevention” strategy). Herein, a choice of model where the take-rate is reproducibly greater than 90% and with consistent growth properties, is critical. Second, treatments may not begin until tumours are just palpable (approx 5 mm diameter, around 60 mm³) (“early-stage” strategy). Herein, one needs to be aware of the possibility of the residual immune system of the host (principally natural killer cell activity) also participating in tumour regression or cures. Finally, treatment may not begin until tumours have reached 8–10 mm diameter (“advanced” stage). In many cases, this is more representative of how chemotherapy will be applied in the clinic, but one needs to evaluate the possibility that either target expression may decrease in larger tumours or that drug uptake/penetration may be compromised because of poor vascularity/increased areas of necrosis. In addition, for rapidly-growing tumours, tumour sizes/volumes may exceed those deemed to be ethically acceptable and result in the early termination of experiments before any delayed drug-induced toxicity may be evident (see below). Issues of animal welfare and ethics within cancer research have recently been considered within the United Kingdom (UK) and published as a set of guidelines [27].

4.5. The test compound

Another critical variable concerns the test agent itself, the way it is administered (route, scheduling, dose) and how it is formulated. In my view, xenograft studies should only be conducted where there is also considerable attention paid to these pharmaceutical (formulation) and pharmacological (pharmacokinetic/pharmacodynamic) principles. Having firmly established that a “lead” molecule possesses potent activity against the target in cell-free assays and within the appropriate tumour cell lines *in vitro*, it is now important to maintain a “pharmaceutical perspective” and consider at this point whether a clinically acceptable formulation of the molecule is attainable. Too many mice have been used in the past with compounds given as coarse suspensions in dimethyl sulphoxide (DMSO) or detergents (e.g. Tweens), vehicles which may not be usable in man. Considerable advances in the development of biocompatible formulations for poorly water-soluble drugs have been developed in recent years (e.g. using cyclodextrin-based systems). However, developing a vehicle is still largely empirical. For particular targets, where chronic administration to patients is the likely clinical scenario based on the understanding of the target, an early evaluation of oral bioavailability may also be appropriate. This may initially be achieved using *in*

in vitro cell line systems such as the colon cancer cell line CaCo2, or directly in rodents by determining pharmacokinetics following oral versus *i.v.* administration of the same dose.

Second, from both a scientific and animal ethical standpoint, xenograft antitumour studies conducted in mice should be limited only to those molecules that possess “adequate” pharmacokinetic properties. For closely related analogues, cassette dosing of mice is a useful tool to rapidly select compounds with optimal pharmacokinetic properties. One should also be prepared to have to compromise on potency against the target in favour of selecting an analogue possessing superior pharmacokinetic properties. The random screening of molecules, especially using *i.p.* drug administration to *i.p.* implanted tumours, a feature of cancer drug screening prevalent in the past, is no longer acceptable. Therefore, a pharmacological approach should be applied whereby only molecules where plasma (or ideally tumour) drug levels known to be required for activity against the target *in vitro* should be tested, and evaluated in xenografts known to possess the target. An elucidation of the effect of dose (and scheduling) should also be conducted thereby providing information on therapeutic index and how the “biologically effective dose” relates to the maximum tolerated dose (MTD). The NCI has recently adopted such an approach with emphasis on pharmacokinetics, mechanism of action and pharmacodynamics post-testing in the 60 cell-line screen and prior to *in vivo* antitumour xenograft studies [28].

4.6. Experimental endpoints

Finally, the choice of endpoint is a critical variable. The most commonly used are those involving measurements of tumour weight (or volume) in untreated or agent vehicle (control) animals versus treated groups on particular days after the start of treatment (e.g. treated [T] versus control [C] values; T/C). A widely used alternative is that using growth delay (GD), the difference in time for treated versus control groups to reach a pre-selected increase in volume (often 3–4-fold) normalised to the starting volume. In addition, a consideration of the growth-rate of control tumours may be applied by determination of specific growth delays (SGD). An unfortunately common feature is for assessments to be made at relatively early time-points and, in particular, before any drug-induced toxicity (body-weight loss, lethargy, even deaths) is evident. Regardless, in all cases, considerably more information may be provided by the provision of full tumour volume versus time curves. In particular, this may indicate whether cytotoxic (tumour shrinkage) or cytostatic (a slowing of tumour growth, but no shrinkage) effects might be expected in the clinic with the test compound (see

example below for the farnesyl transferase inhibitor, R115777).

5. The value (and limitations) of the xenograft model in contemporary cancer drug discovery

Today there is far less focus on the development of further cytotoxics. In addition, there has been a considerable move away from the “black-box” approach to Phase I clinical trials where many agents of unknown mechanisms of action and poorly defined preclinical pharmacokinetics were introduced into the clinic. Does this necessarily mean that the xenograft model is of no further value in contemporary mechanism-directed cancer drug development? I believe that this should be considered on a case-by-case (target-by-target) driven basis. In careful mechanism-based studies, combined with sound pharmacological principles (as described above), then, in my view, the xenograft model remains of great value, both for assisting in the selection of leads for clinical evaluation and for guiding clinical studies (e.g. scheduling, combination strategies).

An example of where xenograft studies have proven useful in selecting and potentially guiding the clinical use of a compound targeting a novel cancer target, is that of G-quadruplex interactive inhibitors of the enzyme telomerase [29]. Herein, the first evidence of *in vivo* activity for such an inhibitor was shown for the trisubstituted acridine BRACO19, when used in combination with paclitaxel in nude mice bearing s.c. A431 epithelial cancer xenografts [30]. In the area of guiding the use of drugs, which *a priori* are not expected to possess significant activity when used alone, but rather will need to be used in combination, our studies with the vascular-targeting drug, 5,6-dimethylxanthenone acetic acid (DMXAA) have demonstrated the potential clinical utility of combination studies with paclitaxel [31].

Certainly, another area where human tumours transplanted in nude or SCID mice remain of considerable value is in the determination of pharmacodynamic markers of drug efficacy, which may then be applied in the clinic. An example is with xenograft studies with the farnesyl transferase inhibitor R115777 [32]. “Typical” xenograft dose response curves for mice bearing a s.c. breast cancer tumour (MCF7) and treated orally with R115777 are shown in Fig. 3a. A point of note in contrast to responses traditionally observed to cytotoxics (e.g. cisplatin, paclitaxel) (Fig. 3c for paclitaxel against a human ovarian carcinoma xenograft) is that the signal transduction inhibitor induced a slowing (cytostatic) effect on tumour growth rather a cytotoxic, tumour shrinkage effect (as for paclitaxel). In addition, as with R115777, chronic (daily) rather than intermittent (e.g. weekly) dosing was required. As emphasised above, knowledge of both the compound mechanism of action

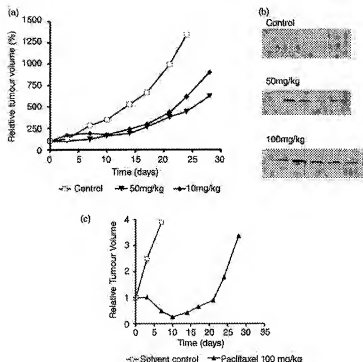


Fig. 3. (a) Tumour dose response curves for the farnesyl transferase inhibitor, R115777, against MCF-7 breast cancer xenografts. Dosing was oral, twice a day for 10 consecutive days. (b) Increase in prelamin A in R115777-treated xenografts (replotted from Ref. 31). (c) Tumour dose response curves for paclitaxel against CH1 ovarian cancer xenografts (dosing was i.p. daily for 5 days).

(molecular target) and pharmacokinetics is essential to guide xenograft experiments and to help predict what effect the compound being tested might be expected to induce. Studies with R115777 and mice bearing MCF-7 breast cancer xenografts showed that the detection of prelamin A may provide a suitable pharmacodynamic marker of response [32] (Fig. 3b).

Another example where xenografts have proven particularly useful in the context of pharmacodynamic markers is in studies of the heat shock 90 inhibitor, 17-allylamino,17-demethoxy geldanamycin (17AAG) [33], now in Phase II clinical trials. For example, it was shown that the detection of an increase in heat shock protein 70 (HSP70) or a decrease in RAF1 in ovarian cancer xenografts (Figs. 4a,b) may represent good pharmacodynamic markers of response in mice or patients receiving 17AAG [34]. In addition, for molecules like 17AAG that possess the potential to disrupt multiple signal transduction pathways, microarray gene expression analyses from treated versus untreated xenografts may also be useful in elucidating mechanisms of action and for identifying potential pharmacodynamic markers of response [35].

Finally, by contrast, it must be borne in mind that there may be instances where the xenograft model is of little or no value. Examples include the use of s.c. xenografts with anti-metastatic strategies as s.c. xeno-

grafts rarely metastasise. Herein, orthotopic models may be more appropriate [25]. Second, drugs (or antibodies) that target tumour vasculature or angiogenesis need to keep in mind that the vasculature being targeted in the xenograft is predominantly of mouse origin. This is of particular relevance with the Antisoma antibody muBC1, which targets an epitope found on human (but not mouse or rat) oncofetal EDB fibronectin [36]. A modification to the xenograft model (at least for prostate cancer) where human vascular endothelium persists in human prostate cancer within implanted human bone fragments [37] may be useful in this context, but is not amenable to large-scale screening. Finally, the use of athymic mice bearing xenografts may be of limited value with naked humanised (or fully human) antibodies. These, such as the Antisoma humanised antibody huHMGF1 (which targets the cell surface antigen, MUC-1) depend upon recruitment of the host's (i.e. mouse) immune response (natural killer cells) to induce antibody-dependent cell-mediated cytotoxicity (ADCC) for activity [38].

6. Summary

During the era of cytotoxic cancer drug discovery, human tumour xenografts s.c. transplanted in athymic

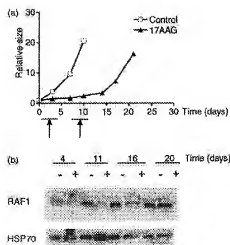


Fig. 4. (a) Tumour growth response curves for the heat shock protein 90 inhibitor, 17-allylamino,17-demethoxy geldanamycin (17AAG) against A2780 ovarian carcinoma xenografts. Dosing was i.p. at 100 mg/kg per day on days 0–4 and 7–11. (b) Immunoblots for HSP70 and RAF1 in treated (+) versus untreated (–) A2780 xenografts on days 4, 11, 16 and 20 after the start of treatment.

mice played a pivotal role in late preclinical agent optimisation and guiding the selection of candidates for Phase I clinical trials. A retrospective review conducted by the NCI for 39 agents for which Phase II activity data were available showed that, where compounds were active in at least one-third of xenograft models tested, there was a statistically significant correlation with activity in at least two tumour types in man [22].

Now, in the post-genomic “molecularly-targeted” era, the case for using human tumour xenografts within a target-driven drug development cascade needs to be justified on a case-by-case basis, keeping in mind both the values and the limitations of the model. These are summarised in Table 2. Limitations include the time and expense (relative to the hollow fibre assay and *in*

vitro testing), ethical issues around animal experimentation, a general lack of metastatic spread from primary s.c. implanted xenografts, and the fact that the stromal compartment of xenografts include vascular endothelial cells that are largely of murine origin.

However, where the particular molecular abnormality of cancer being targeted is shown to be present in a particular human tumour xenograft model (and ideally, shown to be important in the proliferation of that tumour) then s.c.-implanted xenografts still remain of significant value in the cancer drug discovery process. This may be in a therapeutic setting, that is, to obtain *in vivo* ‘proof of principle’ for a particular target and/or to assist in the optimisation of pharmaceutically-tractable molecules. However, the use of mice in *in vivo* anti-tumour studies should be restricted to molecules possessing good pharmaceutical properties where plasma levels above those known to be required for *in vitro* anticancer effects are achievable. At present, in my opinion, it is premature and too much a “leap of faith” to jump directly from *in vitro* activity testing (or even *in silico* methods) to Phase I clinical trials (via preclinical regulatory toxicology). Furthermore, valuable information assisting guidance of the clinical development of a molecule may be obtained; for example, dose-scheduling studies and sequential combination studies with other anticancer drugs. Additionally, and often in parallel with efficacy determinations, the xenograft model is useful in assessing the agent’s pharmacokinetics and pharmacodynamics in that it provides a renewable and readily accessible source of target human tumour cells.

In summary, the human xenograft model in its various guises is here to stay a while longer. Perhaps in another 10 years a similar retrospective analysis as described above by the NCI for cytotoxic cancer drugs, will be applicable to the newer generation “molecularly-targeted” drugs. This may then finally settle the debate as to the value (or otherwise) of the xenograft model in contemporary cancer drug discovery.

Table 2

Summary of values and limitations of the xenograft model

Values

- Validated model for the predictive assessment of cytotoxics—uses human cancer cells with 3-dimensional architecture
- To select lead candidates for clinical evaluation and to optimise scheduling and combination with other drugs to guide clinical development (providing conducted with appropriate consideration to pharmacological principles and pharmacokinetics and pharmacodynamics)
- In vivo* studies of acquired drug resistance and its circumvention

Limitations

- Time (weeks) and expense relative to ‘short-term’ assays such as hollow fibre or *in vitro* cell line testing (plus animal ethics issue relative to *in vitro* testing)
- The s.c. xenograft model generally does not metastasise; not a good model for studying anti-metastatic strategies
- When studying anti-angiogenic or anti-vascular strategies, these components are of murine not human origin
- When studying humanised/human antibodies (immunotherapy) the effector function (e.g. ADCC) being targeted is mouse

ADCC, antibody-dependent cell-mediated cytotoxicity.

References

- Harrison P. Perspective on the history of tumor models. In: Teicher BA, ed. *Tumor Models in Cancer Research*. Totowa, Humana Press Inc, 2002, 3-19.
- Rygaard J, Povlsen CO. Heterotransplantation of a human malignant tumour to the mouse mutant "nude". *Acta Pathologica Microbiol Scand* 1969, 77, 758-760.
- Giovannella BC, Yim SO, Stehlin JS, Williams LJ. Development of invasive tumors in the "hude" mouse after injection of cultured human melanoma cells. *JNCI* 1972, 48, 1531-1533.
- Bosma GC, Custer RP, Bosma MJ. A severe combined immunodeficiency mutation in the mouse. *Nature* 1983, 301, 527-530.
- Donble JA, Bibby MC. Therapeutic index: a vital component in selection of anticancer agents for clinical trial. *Journal National Cancer Institute* 1989, 81, 988-994.
- Hollingshead MG, Alley MC, Camaleri RF, et al. In vivo cultivation of tumor cells in hollow fibers. *Life Sciences* 1995, 57, 131-141.
- Plotman J, Dykes DJ, Hollingshead M, Simpson-Herren L, Alley MC. Human tumor xenograft models in NCI drug development. In: Teicher B, ed. *Anticancer Drug Development Guide: Preclinical Screening, Clinical Trials and Approval*. Humana Press, Totowa, NJ, 1997, 101-125.
- Alley MC, Scudiero DA, Monks A, et al. Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Research* 1988, 48, 589-601.
- Steel GG, Courtenay VD, Peckham MJ. The response to chemotherapy of a variety of human tumour xenografts. *British Journal of Cancer* 1983, 47, 1-13.
- Courtenay VD, Mills J. An in vitro colony assay for human tumours grown in immune suppressed mice and treated in vivo with cytotoxic agents. *British Journal of Cancer* 1978, 37, 261-266.
- Fiebig H-H, Burger AM. Human tumor xenografts and explants. In: Teicher BA, ed. *Tumor Models in Cancer Research*. Totowa, Humana Press Inc, 2002, 113-137.
- Kelland LR, Burgess L, Steel GG. Characterization of four new cell lines derived from human squamous carcinomas of the uterine cervix. *Cancer Research* 1987, 47, 4947-4952.
- Kelland LR, Tonkin KS. Establishment and response to chemotherapy of human cervical carcinoma xenografts. In: Winograd B, Peckham MJ, Pinedo HM, eds. *Human Tumor Xenografts in Anticancer Drug Development*. Berlin, Springer-Verlag, 1988, 57-62.
- Kelland LR, Tonkin KS, Steel GG. A comparison of the in vivo and in vitro radiation response of three human cervix carcinomas. *Radiotherapy & Oncology* 1989, 16, 55-63.
- Harrap KR, Jones M, Siracky J, Pollard LA, Kelland LR. The establishment, characterization and calibration of human ovarian carcinoma xenografts for the evaluation of novel platinum anticancer agents. *Annals Oncology* 1990, 1, 65-76.
- Kelland LR, Jones M, Abel G, et al. Human ovarian carcinoma cell lines and companion xenografts: a disease oriented approach to new platinum anticancer drug development. *Cancer Chemother Pharmacol* 1992, 30, 43-50.
- Kelland LR. New platinum antitumor complexes. *Critical Reviews Oncology/Hematology* 1993, 15, 191-219.
- Jones M, Siracky J, Kelland LR, Harrap KR. Acquisition of platinum drug resistance and platinum cross resistance patterns in a panel of human ovarian carcinoma xenografts. *British Journal of Cancer* 1993, 67, 24-29.
- Kelland LR, Sharp SY, O'Neill CF, et al. Mini-review: discovery and development of platinum complexes designed to circumvent cisplatin resistance. *Journal Inorganic Biochemistry* 1999, 77, 111-115.
- Gore M, Atkinson RJ, Dirix L, et al. ZD0473 phase II monotherapy trial in second-line ovarian cancer. *Proceedings of ASCO* 2001, 20.
- Raynaud FI, Boxall FE, Goddard PM, et al. Cis-amminedichloro(2-methylpyridine) platinum(II) (AMD473), a novel sterically hindered platinum complex: In vivo activity, toxicology and pharmacokinetics in mice. *Clinical Cancer Research* 1997, 3, 2063-2074.
- Johnson JJ, Decker S, Zaharevitz D, et al. Relationships between drug activity in NCI preclinical in vitro and in vivo models and early clinical trials. *British Journal of Cancer* 2001, 84, 1424-1431.
- Casciari JJ, Hollingshead MG, Alley MC, et al. Growth and chemotherapeutic response of cells in a hollow-fiber in vitro solid tumor model. *Journal National Cancer Institute* 1994, 86, 1846-1852.
- Phillips RM, Pearce J, Loadman PM, et al. Angiogenesis in the hollow fiber tumor model influences drug delivery to tumor cells: implications for anticancer drug screening programs. *Cancer Research* 1998, 58, 5263-5266.
- Eccles SA. Models for evaluation of targeted therapies of metastatic disease. In: Teicher BA, ed. *Tumor Models in Cancer Research*. Humana Press, Totowa, 2002, 293-319.
- Corbett TH, Polin L, Roberts BJ, et al. Transplantable syngeneic rodent tumors. In: Teicher BA, ed. *Tumor Models in Cancer Research*. Totowa, Humana Press, 2002, 41-71.
- Workman P, Twentymen P, Balkwill F, et al. United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) guidelines for the welfare of animals in experimental neoplasia (second edition). *British Journal of Cancer* 1999, 77, 1-10.
- Sauville EA, Feigal E. Evolving approaches to cancer drug discovery and development at the National Cancer Institute, USA. *Annals Oncology* 1999, 10, 1287-1291.
- Kelland LR. Telomerase inhibitors: targeting the vulnerable end of cancer? *Anticancer Drugs* 2000, 11, 503-513.
- Gowan SM, Harrison JR, Patterson L, et al. A G-quadruplex-interactive potent small-molecule inhibitor of telomerase exhibiting in vitro and in vivo antitumor activity. *Molecular Pharmacology* 2002, 61, 1154-1162.
- Kelland LR, Rowlinson-Busza G, Griffiths-Johnson D, et al. Single agent activity and potentiation of the antitumor activity of paclitaxel and gemcitabine with the vascular targeting agent 5,6-dimethylxanthone acetic acid (DMXAA) in human tumor xenografts. *Proceedings of AACR* 2003, 44, A3029.
- Kelland LR, Smith V, Valenti M, et al. Preclinical antitumor activity and pharmacodynamic studies with the farnesyl protein transferase inhibitor R15777 in human breast cancer. *Clinical Cancer Research* 2001, 7, 3544-3550.
- Kelland LR, Sharp SY, Rogers PM, Myers TG, Workman P. DT-diphosphor expression and tumor cell sensitivity to 17-allyl-amino-17-demethoxygeldanamycin, an inhibitor of heat shock protein 90. *Journal National Cancer Institute* 1999, 91, 1940-1949.
- Banerji U, Walton M, Raynaud F, et al. Validation of pharmacodynamic endpoints for the HSP90 molecular chaperone inhibitor 17-allyl-amino-17-demethoxygeldanamycin (17AAG) in a human tumor xenograft model. *Proceedings of AACR* 2001, 42.
- Clarke PA, Hostein I, Banerji U, et al. Gene expression profiling of human colon cancer cells following inhibition of signal transduction by 17-allyl-amino-17-demethoxygeldanamycin, an inhibitor of the hsp90 molecular chaperone. *Oncogene* 2000, 19, 4125-4133.
- Midulla M, Verma R, Pignatelli M, et al. Source of oncofetal ED-B containing fibronectin: implications of production by both tumor and endothelial cells. *Cancer Research* 2000, 60, 164-169.
- Nemeth JA, Roberts JW, Mullins CM, Cher ML. Persistence of human vascular endothelium in experimental human prostate cancer bone tumors. *Clinical & Experimental Metastasis* 2000, 18, 231-237.
- Clynes RA, Towers TL, Presta LG, Ravetch JV. Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. *Nature Medicine* 2000, 6, 443-446.

Vesicular Stomatitis Virus: A Potential Therapeutic Virus for the Treatment of Hematologic Malignancy

BRIAN D. LICHTY,¹ DAVID F. STOJDL,¹ REBECCA A. TAYLOR,¹ LEIGH MILLER,¹ IRINA FRENKEL,¹
HAROLD ATKINS,¹ and JOHN C. BELL^{1,2}

ABSTRACT

Certain strains of vesicular stomatitis virus (VSV) have been shown to be oncolytic in a wide variety of solid tumors. In the present study, we tested the leukemolytic properties of VSV using established leukemia cell lines and primary patient material. VSV efficiently killed essentially all leukemic cell lines. In contrast, however, normal clonogenic bone marrow progenitor cells and peripheral blood cells were remarkably refractory to infection by VSV. By exploiting this large difference in susceptibility to infection we successfully purged contaminating leukemic cells from cultures of peripheral blood progenitor cells (PBPC) using VSV. VSV was also able to infect and kill leukemic cells in primary samples taken from patients with multiple myeloma (MM). This study demonstrates the potential utility of VSV in the treatment, both *ex vivo* and *in vivo*, of hematologic malignancies.

OVERVIEW SUMMARY

We have previously proposed the use of vesicular stomatitis virus (VSV) as an oncolytic virus for the treatment of malignant disease. We have also recently reported low toxicity and excellent efficacy in mice with solid tumors after systemic delivery of interferon-inducing VSV mutants. In the present study we have examined the potential utility of VSV as a leukemolytic agent. VSV was able to efficiently kill 11 of 12 lines in a panel of human leukemia cell lines and 3 of 3 primary multiple myeloma patient samples. We also demonstrate the resistance of normal hematopoietic cells to infection and killing by VSV. Also, in this study we have capitalized on this large difference in susceptibility to VSV to purge peripheral blood progenitor cell (PBPC) cultures of contaminating leukemia cells. Taken together, we feel that these strains of VSV are excellent candidates for the treatment of leukemia.

INTRODUCTION

THE INTERFERONS are secreted proteins discovered over 30 years ago on the basis of their antiviral properties (reviewed

in Pestka *et al.*, 1987). Interferon (IFN) acts to inhibit cellular replication and to induce an antiviral state in cells exposed to it (reviewed in Stark *et al.*, 1998). Because of its cytostatic properties it was hoped that IFN would be a valuable cancer therapeutic. Starting in 1970 a number of clinical trials were held to determine the utility of IFN therapy in malignancy. The results of most of these trials were disappointing and today only a handful of cancers are treated with IFN. These include chronic myelogenous leukemia, cutaneous T-cell lymphoma, hairy cell leukemia, melanoma, and renal cell carcinoma (Grander and Einhorn, 1998). Most of the more common malignancies—breast, prostate, colon, lung, stomach, and uterus—do not respond to IFN therapy (Grander and Einhorn, 1998). Additionally, when tumors respond to IFN therapy the response is usually transient because IFN-resistant subclones eventually arise (Grander and Einhorn, 1998). Thus, it would appear that a loss of responsiveness to the cytostatic properties of IFN is a common component of tumorigenesis.

Vesicular stomatitis virus (VSV) is a negative-sense RNA virus. This virus has a broad host cell tropism *in vitro*. In nature, however, the virus most commonly infects farm animals with insect hosts acting as vectors for transmission (Dietzschold *et al.*, 1996). Naturally occurring infections of humans are rare (Fields and Hawkins, 1967) and are generally benign (reviewed

¹Ottawa Regional Cancer Centre Research Laboratories, Ottawa, Ontario, Canada K1H 1C4.

²Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, Ontario Canada, K1N 6N5.

in Lichty *et al.*, 2004). A striking characteristic of VSV is the exquisite sensitivity of the virus to IFN. IFN induces an antiviral state in the host cell that severely inhibits the replication of VSV (Masters and Samuel, 1983). In fact, VSV is commonly used in the laboratory as a biologic assay for the presence of an IFN-induced antiviral state (Julkunen *et al.*, 1982; Salonen and Salmi, 1982; Marquardt *et al.*, 1992).

We have previously reported the extreme sensitivity of human malignant cells to infection and killing by VSV, even in the presence of IFN (Stojdl *et al.*, 2000, 2003). We hypothesize that hematologic malignancies may be an attractive target for oncolytic virus therapy because the target cells should be easily accessed by the therapeutic virus. Additionally there have been many reports of mutations or deletions of members of the interferon regulatory factor (IRF) family of transcription factors in multiple myeloma (MM) and myeloid leukemia (Boulton *et al.*, 1993; Willman *et al.*, 1993; Harada *et al.*, 1994; Ling *et al.*, 1995; Beretta *et al.*, 1996; Iida *et al.*, 1997; Kondo *et al.*, 1997; Haus, 2000; Tzouanopoulos *et al.*, 2002). Conversely, few hematologic malignancies are treated with IFN, outside of chronic myelogenous leukemia, but even there the response tends to be transient and IFN-resistant disease eventually arises (see above). The possibility that myeloid leukemias, in particular, have pre-existing or treatment selected, defects in IFN responsive genes led us to test the susceptibility of acute myeloid lymphoma (AML) cell lines to infection and killing by VSV. In the current study we further assessed the ability of VSV to infect and kill human leukemia cells. We report here the efficient killing of 11 of 12 leukemic cell lines tested. This is in sharp contrast to the remarkable resistance to VSV displayed by normal bone marrow progenitors and peripheral blood cells. We were able to purge peripheral blood progenitor cell (PBPC)/leukemia cell line mixtures of the leukemic cells, demonstrating that these leukemic cells were greater than 1000 times more sensitive to infection and killing by VSV than were the PBPC in the coculture. Additionally, we extend the sensitivity of leukemia cell lines to patient samples and demonstrate that samples from patients with MM are also sensitive to infection and killing by VSV. These results were obtained with IFN-inducing mutants of VSV that are severely attenuated *in vivo* but retain their ability to infect and kill malignant cells because of the defective response to IFN commonly seen in malignant cells (Stojdl *et al.*, 2003; Lichty *et al.*, 2004). This study demonstrates the utility of VSV in the treatment, both *ex vivo* and *in vivo*, of hematologic malignancies.

MATERIALS AND METHODS

Cells and viruses

Cell lines were grown in Iscove's modified Dulbecco's medium (IMDM; Gibco, Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum (FBS) (Gibco). Growth factor-dependent lines (OCI/AML1, OCI/AML4, OCI/AML5, H191 and MO7e) were grown in IMDM supplemented with 10% FBS and 10% 5637-conditioned media. H191 is an AML cell line derived from patient material by H.A. (co-author). Viruses were grown in Vero cells grown on microcarrier beads

in spinner flasks, concentrated on a sodium potassium tartrate isopycnic gradient and titered on Vero cells. The three viral strains used in this study are described elsewhere in detail (Stojdl *et al.*, 2003). Briefly, these viruses consist of the heat-resistant (HR) strain of VSV and the IFN-inducing mutants AV1 and AV2. AV1 and AV2 are naturally occurring mutants displaying a small plaque phenotype on IFN responsive cells and have mutations in the matrix gene (M51R and V221F/S226R mutations in matrix for AV1 and AV2, respectively) (Francoeur *et al.*, 1987).

Recombinant VSV

A recombinant VSV expressing enhanced green fluorescent protein (EGFP) was constructed by subcloning the EGFP open reading frame (ORF) from pEGFP-N1 (Clontech, Palo Alto, CA) into the cloning site introduced between the G and L genes of VSV in the genome vector pVSV-XN (Schnell *et al.*, 1996). This recombinant genome was rescued using standard techniques (Schnell *et al.*, 1996) to generate a replication competent, GFP-expressing VSV clone.

Patient samples

Three patients with MM were included in this study. Diagnosis was based on clinical examination, peripheral blood count, and the morphology and immunophenotype of the leukemic cells. Normal bone marrow cells were obtained from the allogeneic bone marrow transplant donors. Mononuclear cells were collected after centrifugation through a Ficoll gradient. Cryopreserved PBPC samples were obtained by peripheral vein leukapheresis from patients after cytokine therapy to mobilize stem cells. Peripheral blood cells were obtained from normal volunteers and nucleated cells were isolated after red cell lysis. Samples were obtained after informed consent of the patient/donor under a protocol approved by the Ottawa General Hospital Ethics Review Board.

Purging experiments

Purging experiments were carried out by spiking cultures of normal peripheral blood stem cells with leukemic cells that were not cytokine-dependent to a final concentration of 1% leukemic contamination. After infection with virus cells were plated at two serial dilutions per treatment. One dilution series was plated in methylcellulose containing 5% FBS while the other series was plated in Lite Methylcellulose plus Epo (Gencyte, Amherst, NY). Only leukemic progenitors were able to form colonies when plated in methylcellulose containing 5% FBS as pure PBPC samples failed to form any colonies. The methylcellulose cultures were examined after 10 days and the number of leukemic precursors (colony count in 5% serum) and the number of normal precursors (colony count in Gencyte methylcellulose minus colony count in 5% serum) was determined for each treatment.

Infections

Infections of suspension cells were carried out in small volumes of media at cell concentrations greater than $1 \times 10^6/\text{ml}$

for 45 minutes at 37°C with occasional mixing and then plated into culture dishes in the appropriate media.

Colony assays

Bone marrow and PBPC colony assays were performed by plating duplicate serial dilutions in Lite Methylcellulose plus Epo (Gencyte) in 35-mm dishes followed by incubation at 37°C for 10 to 14 days. Colonies were scored as CFU-E (colony forming units-erythroid) or CFU-GM (colony forming units-granulocyte/macrophage). Cell line colony assays were performed by plating duplicate serial dilutions in methylcellulose plus 10% FBS. For cytokine-dependent cell lines (see above) 10% 5637-conditioned media was included.

Flow cytometry

The viability of unfixed cells was assessed by staining with propidium iodide followed by flow cytometric analysis. Detection of infected cells was determined by enumeration of green fluorescent cells after infection with a GFP-expressing VSV. Peripheral blood populations were identified after staining with anti-CD3 (T cell), anti-CD19 (B cell), anti-CD14 (monocytes), and anti-CD13 (neutrophils). Plasma/myeloma cells in the samples from patients with MM were identified using anti-CD138 (all antibodies from Becton-Dickinson, Franklin Lakes, NJ).

RESULTS

VSV killing of clonogenic leukemia precursors

The leukemolytic properties of VSV were evaluated using a panel of leukemia cell lines and four distinct virus strains. Essentially, cells were infected at a multiplicity of infection (MOI) of 1.0 plaque-forming units per cell (pfu/cell) and then grown

in a methylcellulose suspension to evaluate viability. The four different virus strains used were the heat-resistant or HR variant of VSV Indiana, a recombinant version of HR harboring the GFP gene and the IFN-inducing mutants AV1 and AV2 (Stojdl *et al.*, 2003). In our earlier nude mouse studies, we had shown that while the HR strain of Indiana has good oncolytic properties, maximum therapeutic benefit was obtained when this virus was combined with IFN. On the other hand, the IFN-inducing mutants AV1 and AV2, effectively kill tumor cells lacking an IFN response but are highly attenuated for growth in normal tissues even in the absence of exogenously added IFN (Stojdl *et al.*, 2003).

As can be seen in Table 1, most of the 12 cell lines tested in this way were sensitive to VSV with a reduction of as much as 5 logs in colony forming ability compared to mock-infected cultures. VSV-mediated oncolysis extended to a wide variety of leukemia types, although cell lines of lymphoid origin were generally more resistant to the virus, especially to the IFN-inducing mutants AV1 and AV2. Both the HR strain and its derivative engineered to express GFP, were equally potent at killing leukemia cells indicating that the addition of an extra gene to the virus did not markedly compromise its oncolytic activity.

Flow cytometric analysis of infected leukemia cell lines

While the colony-forming assay is a robust means of evaluating virus-mediated cell killing, it is limited. We sought a second method to evaluate VSV infection and killing of malignant hematopoietic cells. To this end we used flow cytometry following propidium iodide (PI) staining of unfixed cells to determine viability after infection (Table 2). This method allowed us to analyze cell lines that failed to form countable colonies in methylcellulose such as NCI/H929 and OCI/AML4. As ex-

TABLE 1. KILLING OF LEUKEMIC CELL LINES BY VESICULAR STOMATITIS VIRUS AS ASSESSED BY COLONY COUNTS

Cell line	Average colony count per 10 ⁵ /cells plated				
	Mock	HR	AV1	AV2	GFP
LY-8 (B-cell lymphoma)	44927 ± 11930	3079 ± 2378	5560 ± 2436	3693 ± 1101	120 ± 35
LY-18 (B-cell lymphoma)	5254 ± 1740	3 ± 3	44 ± 20	311 ± 499	1 ± 1
SR (large cell lymphoma)	18738 ± 6375	0 ± 0	1 ± 1	0 ± 0	0 ± 0
MOLT-4 (acute lymphoblastic)	56977 ± 31602	104 ± 72	513 ± 71	899 ± 608	358 ± 34
Jurkat (acute lymphoblastic)	68444 ± 10584	0 ± 0	0 ± 0	0 ± 0	0 ± 0
OCI/My10 (myeloma)	35827 ± 11724	1 ± 2	58 ± 61	10 ± 8	0 ± 0
K562 (chronic myeloid)	44913 ± 17646	0 ± 0	3 ± 3	8 ± 6	0 ± 0
MO7e (megakaryoblastic)	16131 ± 5092	8 ± 7	43 ± 6	3 ± 3	1 ± 1
OCI/AML1 (acute myeloid)	32692 ± 24561	1 ± 1	1 ± 1	4 ± 3	0 ± 0
OCI/AML3 (acute myeloid)	13600 ± 6699	0 ± 0	0 ± 0	0 ± 0	0 ± 0
OCI/AML5 (acute myeloid)	66520 ± 7429	0 ± 0	0 ± 0	0 ± 0	0 ± 0
HI91 (acute myeloid)	36631 ± 12552	0 ± 0	3 ± 2	14 ± 5	0 ± 0

Leukemic cells were infected with the indicated strain (HR, heat-resistant; GFP, recombinant GFP-expressing attenuated virus [AV1] and attenuated virus [AV2]) of VSV at an MOI of 1.0. Serial dilutions (10², 5 × 10², and 10³ for uninfected cultures and 10³, 10⁴, and 10⁵ for infected cultures) were plated in methylcellulose at 72 hr postinfection and counted ~10 days later. Mock, HR, and AV3 counts represent four independent experiments each plated in triplicate; AV1 counts derive from three independent experiments each done in triplicate and the GFP counts are from one experiment done in duplicate.

VSV, vesicular stomatitis virus; MOI, multiplicity of infection.

TABLE 2. FLOW CYTOMETRIC ANALYSIS OF HUMAN LEUKEMIA CELL LINES INFECTED WITH VESICULAR STOMATITIS VIRUS

A: Lymphoid leukemia cell lines

<hr/>					
LY-8 (B-cell lymphoma)					
	VSV (–)	HR	AV2	GFP	GFP +
24 hr	11.8	ND	12.8	55.9	49.9
48 hr	7.1	ND	58.4	66.1	47.5
LY-18 (B-cell lymphoma)					
	VSV (–)	HR	AV2	GFP	GFP +
24 hr	6.5	ND	15.2	60.6	55.1
48 hr	3.0	ND	53.7	85.1	44.7
SR (large cell lymphoma)					
	VSV (–)	HR	AV2	GFP	GFP +
24 hr	21.3	63.5	51.7	27.3	34.0
48 hr	25.1	90.2	92.1	84.5	7.1
MOLT-4 (T-cell acute lymphoid leukemia)					
	VSV (–)	HR	AV2	GFP	GFP +
24 hr	9.4	32.2	21.0	20.8	66.4
48 hr	6.7	75.9	65.5	59.8	59.5
Jurkat (T-cell acute lymphoid leukemia)					
	VSV (–)	HR	AV2	GFP	GFP +
24 hr	6.7	26.5	53.4	38.5	90.1
48 hr	11.48	84.8	98.1	93.2	94.8
OCI/My10 (myeloma)					
	VSV (–)	HR	AV2	GFP	GFP +
24 hr	20.2	ND	77.3	88.6	10.7
48 hr	17.9	ND	87.7	89.3	3.6
NCI-H929 (myeloma)					
	VSV (–)	HR	AV2	GFP	GFP +
24 hr	5.1	ND	8.4	97.4	1.5
48 hr	5.4	ND	98.9	99.1	0.0

B: Myeloid leukemic cell lines

<hr/>					
OCI/AML1 (acute myeloid leukemia)					
	VSV (–)	HR	AV2	GFP	GFP +
48 hr	7.6	63.6	73.8	45.8	87.6
72 hr	3.1	94.0	85.6	95.4	60.1
OCI/AML3 (acute myeloid leukemia)					
	VSV (–)	HR	AV2	GFP	GFP +
24 hr	1.8	56.4	65.7	65.2	76.6
48 hr	2.1	98.9	98.5	97.3	25.3
OCI/AML4 (acute myeloid leukemia)					
	VSV (–)	HR	AV2	GFP	GFP +
24 hr	15.0	17.3	15.6	15.4	0.7
48 hr	15.5	33.4	16.3	20.5	14.4
OCI/AML5 (acute myeloid leukemia)					
	VSV (–)	HR	AV2	GFP	GFP +
24 hr	5.1	57.1	60.6	57.4	93.6
48 hr	11.7	98.4	96.1	98.1	96.7
H191 (acute myeloid leukemia)					
	VSV (–)	HR	AV2	GFP	GFP +
24 hr	7.6	27.0	41.9	32.3	87.0
K562 (chronic myeloid leukemia)					
	VSV (–)	HR	AV2	GFP	GFP +
24 hr	13.5	22.6	57.8	21.7	66.7
48 hr	ND	60.0	78.6	51.6	78.3
MO7e (megakaryoblastic leukemia)					
	VSV (–)	HR	AV2	GFP	GFP +
24 hr	27.2	44.3	46.4	60.2	89.9
48 hr	26.0	94.1	93.1	94.1	94.1
72 hr	39.0	95.1	93.0	96.4	94.7

Cell lines were infected at an MOI of 1.0, with the indicated strain of VSV, for various periods of time. Unfixed cells were stained with propidium iodide and assessed by flow cytometry. Numbers shown are percent dead or percent GFP-positive where noted. Percentages are based on 10,000 cells counted per treatment.

MOI, multiplicity of infection; VSV, vesicular stomatitis virus; GFP, green fluorescent protein; HR, heat-resistant; AV2, attenuated virus 2.

pected, there was good agreement between methylcellulose colony assay and the flow cytometric analysis, although in general, flow cytometry tended to underestimate the killing ability of the virus. In experiments using our recombinant VSV-GFP virus, it was possible to monitor the progression of the viral infection. Shortly after infection, leukemic cells began to express GFP but as the infection progressed and cells began to die (as measured by PI uptake) GFP expression was lost from some of the cell lines. For instance, OCI/AML3 and SR were efficiently killed and had lost GFP expression by 48 hr postinfection while K562 and Mo7e were also killed efficiently but they retained GFP fluorescence. The reason for this variability is not clear but perhaps reflects differences in the death pathway for these different cell types. All three strains of the virus displayed efficient infection and killing of most cell lines although the IFN-

inducing mutant AV2 displayed slower growth kinetics than the wild-type strains. Overall the combination of colony assay and flow cytometry provides convincing evidence for the killing of lymphoma, acute lymphoblastic, acute myeloid and chronic myeloid leukemia cell lines.

Normal bone marrow progenitors are refractory to VSV infection

In contrast to the exquisite sensitivity of leukemic cell lines to VSV infection, normal clonogenic bone marrow progenitors are largely unaffected by the virus. As an example, in the experiments shown in Figure 1A, bone marrow from normal donors was infected with the HR-GFP strain of Indiana virus at an MOI of 5 pfu/cell and the infection allowed to proceed

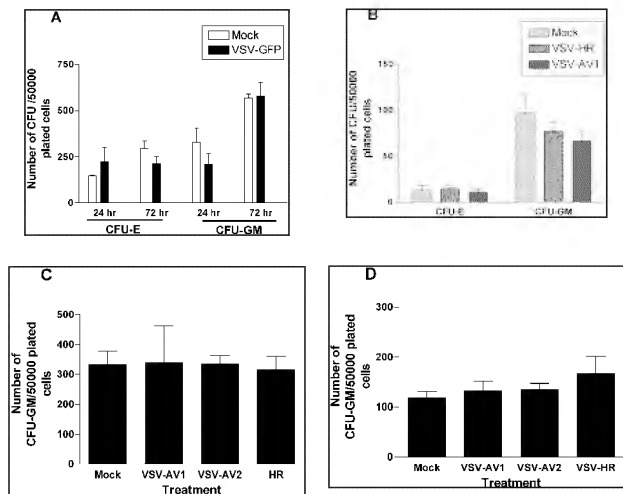


FIG. 1. Normal bone marrow progenitors are resistant to vesicular stomatitis virus (VSV). **A:** A normal bone marrow was infected at a multiplicity of infection (MOI) of 5.0 with VSV-green fluorescent protein (GFP) and at the indicated time points samples were serially diluted and plated in methylcellulose in duplicate. **B:** A second normal bone marrow sample was infected with each of the indicated viruses at an MOI of 10.0 and at 24 hr postinfection samples were serially diluted in triplicate and plated in methylcellulose. **C:** A third normal bone marrow sample was infected with each of the indicated viruses at an MOI of 1.0 and at 1 hr postinfection samples were serially diluted and plated in methylcellulose in duplicate. **D:** A fourth normal bone marrow sample was infected with each of the indicated viruses at an MOI of 1.0 and at 1 hr postinfection samples were serially diluted in triplicate and plated in methylcellulose. In each case colonies were counted after 14 days. Number of colonies shown is per 5×10^4 cells plated. CFU-E, colony forming units-erythroid; CFU-GM, colony forming units-granulocyte/macrophage.

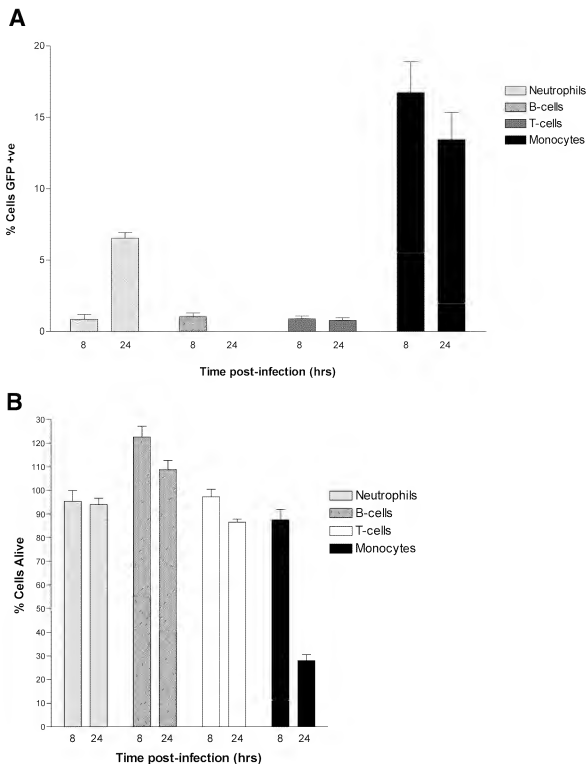


FIG. 2. Infection of normal peripheral blood cells with vesicular stomatitis virus (VSV). White blood cells were isolated from the peripheral blood of a normal volunteer and infected with VSV-green fluorescent protein (GFP) at a multiplicity of infection (MOI) of 10 plaque-forming units (PFU) per cell. **A:** Percentage of cells in each population expressing GFP at 8 and 24 hours post-infection. **B:** Viability of each subset after infection normalized to uninfected population.

for some 72 hr. At this time, the culture was plated in methylcellulose and the number of colony forming cells (CFCs) determined 14 days later. It is clear from Figure 1 that normal progenitors are unaffected by VSV infection. In a second series of experiments (Fig. 1B), using the IFN-inducing virus strains (AV1 and AV2) and the parental HR strain there was no significant difference in the plating efficiency of normal progenitors following VSV infection. Similar results were obtained for two additional bone marrow samples (Fig. 1C and 1D).

Normal lymphocytes and neutrophils are resistant to infection by VSV

To determine the degree to which normal peripheral blood cells could be infected with VSV white blood cells from five normal volunteers were infected with a recombinant VSV expressing GFP at an MOI of 10 and flow cytometry was used to determine which cell populations, if any, became infected *in vitro*. Data for a representative sample is shown in Figure 2. These analyses demonstrated that lymphocytes were very resistant to infection even at this high MOI. There was some infection of neutrophils by 24 hr (range, 4.1% to 7.9%) and monocytes were somewhat susceptible showing infection at both time points (range at 8 hr, 3.4% to 17.2% and range at 24 hr, 13.3%

to 35.1%). It should be noted however that CFU-GM are resistant, therefore this susceptibility appears to be confined to mature, peripheral monocytes.

Purging of leukemia cells from mixed cultures with VSV

In the clinical setting, peripheral blood stem cells (PBSC) are used as a source of hematopoietic stem cells to perform autotransplants for patients with leukemia and contaminating leukemic cells are likely problematic (Deisseroth *et al.*, 1994; Rill *et al.*, 1994; Heslop *et al.*, 1996). The extreme sensitivity of leukemic cells to VSV infection coupled with the remarkable resistance to the virus displayed by bone marrow progenitors led us to test the ability of the virus to purge PBPC cultures of contaminating leukemic cells. To this end, leukemia cell lines were mixed with PBPC and infected with VSV. Infected cultures were serially diluted and plated in methylcellulose containing growth factors to support normal CFCs or methylcellulose containing 5% FBS to exclusively support growth of leukemic colonies (Table 3). PBPC progenitors were also resistant to killing by the attenuated AV2 strain of VSV but were affected by infection with the HR strain. When cultures containing leukemic cells were infected with the HR and AV2 strains there was a complete ablation of leukemic cells and a

TABLE 3. LEUKEMIA CELLS ARE MORE THAN 1000 TIMES MORE SENSITIVE TO VESICULAR STOMATITIS VIRUS THAN ARE PERIPHERAL PROGENITOR CELLS

	Colonies per 10 ⁵ cells plated		
	CFU-E	CFU-GM	
PBPC			
Mock	282 ± 34	193 ± 48	
VSV-HR	113 ± 21	79 ± 31	
AV2	203 ± 28	165 ± 45	
PBPC plus 1% MOLT-4			
	CFU-E	CFU-GM	Leukemic
Mock	ND	ND	1140 ± 48
VSV-HR	98 ± 20	58 ± 26	0
AV2	168 ± 26	143 ± 41	0
PBPC plus 1% OCI/AML3			
	CFU-E	CFU-GM	Leukemic
Mock	ND	ND	1243 ± 50
VSV-HR	49 ± 14	40 ± 22	0
AV2	123 ± 22	112 ± 37	0

Peripheral blood from a patient treated with cytokines to mobilize stem cells was infected with VSV-HR and VSV-AV2. In addition cultures were spiked with 1% leukemia cells and infections were carried out in parallel. Cultures were infected at an MOI of 1.0 and 24 hours post-infection samples were plated in methylcellulose + 5% FBS (to enumerate leukemic colonies) or in Lite Methylcellulose plus Epo (Gencyte, Amherst, NY, to enumerate leukemic plus normal colonies, the number of normal colonies indicated represents the difference between Gencyte and 5% FBS). When mock infected the leukemia-contaminated cultures produced copious colonies in the presence of cytokines making the number of normal colonies present difficult to accurately enumerate.

VSV, vesicular stomatitis virus; HR, heat-resistant; AV2, attenuated virus 2; MOI, multiplicities of infection; FBS, fetal bovine serum.

variable but relatively small reduction in progenitor numbers. There was a reduction in PBPC progenitors of less than 50% when the cocultures were infected with AV2 but a greater than 1000-fold reduction in leukemia progenitors. The apparently greater impact of the virus on normal progenitors in the context of these mixed cultures may have been the result of replication of the virus in the leukemic cells or an indirect effect of the leukemic cells dying in these cultures. These experiments clearly demonstrate that VSV is able to specifically kill leukemic cells in mixed cultures with relatively minimal impact on normal progenitor stem cells.

VSV kills myeloma cells from primary patient samples

In order to test the ability of VSV to infect and kill primary malignant cells we obtained peripheral blood samples from three patients with MM. We chose patients with MM because we had access to several samples, we had seen good killing of myeloma cell lines, and the expression of CD138 by myeloma cells made the malignant cell population easily identified by flow cytometry. Samples from these patients were infected with wild-type or IFN-inducing mutant VSV strains and the cultures were watched for 8 days postinfection. Figure 3 demonstrates the reduction in viable CD138⁺ cells after infection with any of these three VSV strains. While each patient had a high number of CD138⁺ cells in their peripheral blood infection with VSV was able to completely ablate these cells in one patient sample and significantly reduce the number of CD138⁺ cells in the other two patient samples following a single dose of VSV.

Taken together these analyses indicate that VSV may be a useful therapeutic in multiple myeloma.

DISCUSSION

In earlier studies, we reported the increased sensitivity of malignant cells to infection and killing by VSV. This virus is extremely sensitive to the innate IFN mediated antiviral response, which appears to be commonly dysregulated in cancerous cells. Here, we have extended this analysis to a range of leukemic cell lines and to primary leukemia patient samples. We demonstrate that most leukemic cell lines tested are highly susceptible to infection and killing by VSV, including IFN-inducing mutants that are attenuated *in vivo* (Stojdl *et al.*, 2003). In these and other experiments we have been struck by the extreme resistance of normal bone marrow progenitors to VSV infection. While in our hands, VSV is able to productively infect most cell types, it is clear that bone marrow stem cells and normal peripheral blood lymphocytes have active antiviral programs that rapidly blunt virus infections. Indeed in other studies, we have found that bone marrow stem cells resist infection by laboratory strains of reovirus, Newcastle disease virus, measles virus, and mumps virus (data not shown). The susceptibility to infection displayed by normal monocytes is not surprising as these cells have been demonstrated to be susceptible to viral infection by others (Jahn *et al.*, 1999) and may be important in antigen presentation by these cells. The fact that the

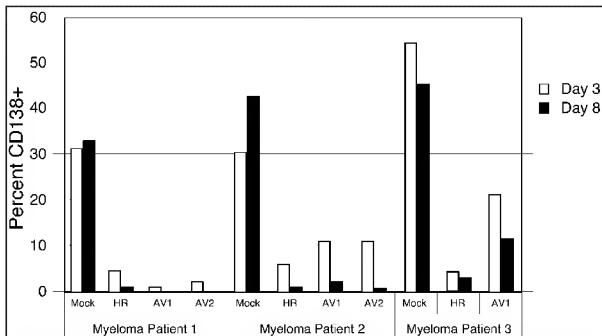


FIG. 3. *In vitro* infection and killing of samples from patients with primary multiple myeloma. Peripheral blood samples from 3 patients with multiple myeloma were infected *in vitro* with the indicated strains of vesicular stomatitis virus (VSV) at a multiplicity of infection (MOI) of 10 plaque-forming units (PFU) per cell. Samples were collected 3 and 8 days postinfection and analyzed by flow cytometry after staining for CD138. 10,000 events were recorded per sample per time point; percentages of viable cells that were CD138 positive are indicated.

progenitors for this mature cell population, namely normal CFU-GM, are resistant to VSV suggests that this cell population will be replaced by the patient's bone marrow after treatment.

The results presented here suggest that a common feature of leukemic cells appears to be the loss of at least portions of an innate antiviral response. In support of this idea, a number of reports of defects, deletions and mutations to genes involved in the IFN response in myeloproliferative disease. For instance, IRF-1 is known to have the properties of a tumor suppressor gene in that while it plays a pivotal role in the normal IFN response to virus infection, (reviewed in Tanaka and Taniguchi, 2000) it is located at 5q31, a region frequently deleted in myeloid leukemias (Van den Berghe *et al.*, 1985; Boultonwood *et al.*, 1993; Willman *et al.*, 1993). Similarly, mutations to the PKR gene (Beretta *et al.*, 1996; Abraham *et al.*, 1998) have been reported in hematologic malignancies. As well, dysregulation of MUM1/IRF4 has also been reported in hematologic malignancy, particularly in MM (Iida *et al.*, 1997; Yoshida *et al.*, 1999; Chesi *et al.*, 2000; Tsuboi *et al.*, 2000; Yamada *et al.*, 2001). Consistent with these observed aberrations in the IFN signaling pathway is the fact that only a handful of hematologic malignancies respond to IFN therapy (Gander and Einhorn, 1998) and that these frequently evolve to nonresponsive cancers.

The samples from patients with MM examined in this study displayed slower kinetics of killing after viral infection than the established cell lines examined although the degree of killing of primary leukemia cells is comparable to that reported for oncolytic adenovirus (Medina *et al.*, 1999) and reovirus (Alain *et al.*, 2002; Thirukkumaran *et al.*, 2003). As well, it should be noted that the degree of killing appears to be underestimated when assayed by flow cytometry at a given time point. We are currently acquiring additional patient samples in order to determine whether MM samples are generally sensitive to VSV and to determine whether other leukemias, particularly AMLs, are also susceptible. Importantly, the enormous difference in susceptibility to VSV displayed by normal progenitors and leukemic cells suggests that the virus can be used to selectively kill leukemic cells in a mixed population. Our purging experiments support this notion. We are continuing these studies in order to determine whether there are particular classes of myeloid leukemia wherein VSV therapy might be particularly effective. We are also interested in the susceptibility of MM cells to infection and killing by VSV (and other oncolytic viruses), which is in sharp contrast to the complete resistance to infection displayed by most other lymphoid cells. Indeed this study may indicate that there is a defect in antiviral pathways in MM cells that render them particularly sensitive to infection and killing by VSV.

There have been concerns voiced by some regarding the use of replicating viral vectors in the clinical setting. We have shown that the IFN-inducing mutants used in this study are significantly attenuated *in vivo* and that they induce a cytokine cloud in the host animal effectively protecting the recipient from any wild-type revertants present in the inoculum (Stojdl *et al.*, 2003). Several groups are now proposing to use VSV as a therapeutic virus and preclinical testing combined with a large body of historical data regarding natural infections of humans with this virus indicate that even the wild-type strains of this virus

generally produce benign infections in naive humans (reviewed in Lichty *et al.*, 2004).

The field of oncolytic virus therapy is expanding rapidly (Bell *et al.*, 2002) but in most cases these therapies are directed against solid tumors. However, efficient killing of leukemia and lymphoma cells has now been demonstrated for measles (Grote *et al.*, 2001; Peng *et al.*, 2001), adenovirus (Medina *et al.*, 1999; Strair *et al.*, 2002), reovirus (Alain *et al.*, 2002; Thirukkumaran *et al.*, 2003) and VSV (Stojdl *et al.*, 2000; Dummer *et al.*, 2001). In particular the degree of killing reported here for MM patient samples compares favourably with that seen for oncolytic measles and reovirus (Peng *et al.*, 2001, 2003; Thirukkumaran *et al.*, 2003). VSV is able to infect virtually all cell types but its replication is blocked in cells that have an intact innate antiviral system, especially if these cells have been exposed to IFN. Taken as a whole these observations and the results reported in this study point to the utility of VSV as a leukemolytic agent for the *in vivo* and *ex vivo* treatment of hematologic malignancy.

ACKNOWLEDGMENTS

This study was supported by grants to J.C.B. from the Canadian Institutes of Health Research and the Leukemia Research Fund of Canada. B.D.L. was supported by a Terry Fox Postdoctoral Fellowship from the National Cancer Institute of Canada.

Thank you to Shane Knowles for technical assistance and to Drs. Richard Wells and Ken Garson for critical review of the manuscript.

REFERENCES

- ABRAHAM, N., JARAMILLO, M.L., DUNCAN, P.I., METHOT, N., ICELY, P.L., STOJDL, D.F., BARBER, G.N., and BELL, J.C. (1998). The murine PKR tumor suppressor gene is rearranged in a lymphocytic leukemia. *Exp. Cell Res.* **244**, 394-404.
- ALAIN, T., HIRASAWA, K., PON, K.J., NISHIKAWA, S.G., URBANSKI, S.J., AUER, Y., LUJDER, J., MARTIN, A., JOHNSTON, R.N., JANOWSKA-WIECZOREK, A., LEE, P.W., and KOSAKOWSKA, A.E. (2002). Reovirus therapy of lymphoid malignancies. *Blood* **100**, 4146-4153.
- BELL, J.C., GARSON, K.A., LICHTY, B.D., and STOJDL, D.F. (2002). Oncolytic viruses: programmable tumour hunters. *Curr. Gene Ther.* **2**, 243-254.
- BERETTA, L., GABBAY, M., BERGER, R., HANASH, S.M., and SONENBERG, N. (1996). Expression of the protein kinase PKR in modulated by IRF-1 and is reduced in 5q- associated leukemias. *Oncogene* **12**, 1593-1596.
- BOULTWOOD, J., FIDLER, C., LEWIS, S., MACCARTHY, A., SHERIDAN, H., KELLY, S., OSCIER, D., BUCKLE, V.J., and WAINSCOT, J.S. (1993). Allelic loss of IRF1 in myelodysplasia and acute myeloid leukemia: retention of IRF1 on the 5q- chromosome in some patients with the 5q- syndrome. *Blood* **82**, 2611-2616.
- CHESE, M., KUEHL, W.M., and BERGSAGEL, P.L. (2000). Recurrent immunoglobulin gene translocations identify distinct molecular subtypes of myeloma. *Ann. Oncol.* **11**(Suppl 1), 131-135.

- DEISSEROTH, A.B., ZU, Z., CLAXTON, D., HANANIA, E.G., FU, S., ELLERSON, D., GOLDBERG, L., THOMAS, M., JANICEK, K., and ANDERSON, W.F. (1994). Genetic marking shows that Ph⁺ cells present in autologous transplants of chronic myelogenous leukemia (CML) contribute to relapse after autologous bone marrow in CML. *Blood* **83**, 3068–3076.
- DIETZSCHOLD, B., RUPPRECHT, C.E., FU, Z.F., and KO-PROWSKI, H. (1996). Rhabdoviruses. In *Fields Virology*. B.N. Fields, D.M. Knipe, and P.M. Howley, eds. (Lippincott-Raven, Philadelphia, PA) pp. 341–346.
- DUMMER, R., DOBBELING, U., GEERTSEN, R., WILLERS, J., BURG, G., and PAVLOVIC, J. (2001). Interferon resistance of cutaneous T-cell lymphoma-derived clonal T-helper 2 cells allows selective viral replication. *Blood* **97**, 523–527.
- FIELDS, B.N., and HAWKINS, K. (1967). Human infection with the virus of vesicular stomatitis during an epizootic. *N. Engl. J. Med.* **277**, 989–994.
- FRANCOEUR, A.M., POLIQUIN, L., and STANNERS, C.P. (1987). The isolation of interferon-inducing mutants of vesicular stomatitis virus with altered viral P function for the inhibition of total protein synthesis. *Virology* **160**, 236–245.
- GRANDER, D., and EINHORN, S. (1998). Interferon and malignant disease—How does it work and why doesn't it always? *Acta Oncol.* **37**, 331–338.
- GROTE, D., RUSSELL, S.J., CORNU, T.L., CATTANEO, R., VILLE, R., POLAND, G.A., and FIELDING, A.K. (2001). Live attenuated measles virus induces regression of human lymphoma xenografts in immunodeficient mice. *Blood* **97**, 3746–3754.
- HARADA, H., KONDO, T., OGAWA, S., TAMURA, T., KITAGAWA, M., TANAKA, N., LAMPHIER, M.S., HIRAI, H., and TANIGUCHI, T. (1994). Accelerated exon skipping of IRF-1 mRNA in human myelodysplasia/leukemia: A possible mechanism of tumor suppressor inactivation. *Oncogene* **9**, 3313–3320.
- HAUS, O. (2000). The genes of interferons and interferon-related factors: localization and relationships with chromosome aberrations in cancer. *Arch. Immunol. Ther. Exp. (Warsz)* **48**, 95–100.
- HESLOP, H.E., ROONEY, C.M., RILL, D.R., KRANCE, R.A., and BRENNER, M.K. (1996). Use of gene marking in bone marrow transplantation. *Cancer Detect. Prev.* **20**, 108–113.
- IIDA, S., RAO, P.H., BUTLER, M., CORRADINI, P., BOCCADORO, M., KLEIN, B., CHAGANTI, R.S., and DALLA-FAVERA, R. (1997). Deregulation of MUM1/IRF4 by chromosomal translocation in multiple myeloma. *Nat. Genet.* **17**, 226–230.
- JAHN, G., STENGLEIN, S., RIEGLER, S., EINSELE, H., and SINZGER, C. (1999). Human cytomegalovirus infection of immature dendritic cells and macrophages. *Intervirology* **42**, 365–372.
- JULKUNEN, L., LINNAVUORI, K., and HOVI, T. (1982). Sensitive interferon assay based on immunoenzymatic quantification of viral antigen synthesis. *J. Virol. Methods* **5**, 85–91.
- KONDO, T., MINAMINO, N., NAGAMURA-INOUE, T., MATSUMOTO, M., TANIGUCHI, T., and TANAKA, N. (1997). Identification and characterization of nucleophosmin/B23/nucleolin which binds the anti-oncogenic transcription factor IRF-1 and manifests oncogenic activity. *Oncogene* **15**, 1275–1281.
- LICHTY, B.D., POWER, A.T., STOIDL, D.F., and BELL, J.C. (2004). Vesicular stomatitis virus: Re-inventing the bell. *Trends Mol. Med.* **10**, 210–216.
- LINGE, C., GEWERT, D., ROSSMANN, C., BISHOP, J.A., and CROWE, J.S. (1995). Interferon system defects in human malignant melanoma. *Cancer Res.* **55**, 4099–4104.
- MARQUARDT, J., HEINZ, H., HEYMER, J., DEEGEN, E., ADOLF, G.R., and LEIBOLD, W. (1992). A method for the assay of “difficult” interferons exemplified with recombinant equine interferon-beta 1. *J. Interferon Res.* **12**, 83–85.
- MASTERS, P.S., and SAMUEL, C.E. (1983). Mechanism of interferon action: Inhibition of vesicular stomatitis virus replication in human amnion U cells by cloned human leukocyte interferon. I. Effect on early and late stages of the viral multiplication cycle. *J. Biol. Chem.* **258**, 12019–12025.
- MEDINA, D.J., SHEAY, W., GOODELL, L., KIDD, P., WHITE, E., RABSON, A.B., and STRAIR, R.K. (1999). Adenovirus-mediated cytotoxicity of chronic lymphocytic leukemia cells. *Blood* **94**, 3499–3508.
- PENG, K.W., AHMANN, G.J., PHAM, L., GREIFF, P.R., CATTANEO, R., and RUSSELL, S.J. (2001). Systemic therapy of myeloma xenografts by an attenuated measles virus. *Blood* **98**, 2002–2007.
- PENG, K.W., DONOVAN, K.A., SCHNEIDER, U., CATTANEO, R., LUST, J.A., and RUSSELL, S.J. (2003). Oncolytic measles viruses displaying a single-chain antibody against CD38, a myeloma cell marker. *Blood* **101**, 2557–2562.
- PESTKA, S., LANGER, J.A., ZOON, K.C., and SAMUEL, C.E. (1987). Interferons and their actions. *Annu. Rev. Biochem.* **56**, 727–777.
- RILL, D.R., SANTANA, V.M., ROBERTS, W.M., NILSON, T., BOWMAN, L.C., KRANCE, R.A., HESLOP, H.E., MOEN, R.C., IHLE, J.N., and BRENNER, M.K. (1994). Direct demonstration that autologous bone marrow transplantation for solid tumors can return a multiplicity of tumorigenic cells. *Blood* **84**, 380–383.
- SALONEN, R., and SALMI, A. (1982). An interferon assay based on yield reduction of vesicular stomatitis virus antigen measured by enzyme immunoassay. *J. Virol. Methods* **5**, 143–149.
- SCHNELL, M.J., BUONOCORE, L., WHITT, M.A., and ROSE, J.K. (1996). The minimal conserved transcription stop-start signal promotes stable expression of a foreign gene in vesicular stomatitis virus. *J. Virol.* **70**, 2318–2323.
- STARK, G.R., KERR, I.M., WILLIAMS, B.R., SILVERMAN, R.H., and SCHREIBER, R.D. (1998). How cells respond to interferons. *Annu. Rev. Biochem.* **67**, 227–264.
- STOIDL, D.F., LICHTY, B.D., KNOWLES, S., MARIUS, R., ATKINS, H., SONENBERG, N., and BELL, J.C. (2000). Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus. *Nat. Med.* **6**, 821–825.
- STOIDL, D.F., LICHTY, B.D., TENOVER, B.R., PATERSON, J.M., POWER, A.T., KNOWLES, S., MARIUS, R., REYNARD, J., POLIQUIN, L., ATKINS, H., BROWN, E.G., DURBIN, R.K., DURBIN, J.E., HISCOTT, J., and BELL, J.C. (2003). VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents. *Cancer Cell* **4**, 263–275.
- STRAIR, R.K., SHEAY, W., GOODELL, L., WHITE, E., RABSON, A.B., and MEDINA, D.J. (2002). Adenovirus infection of primary malignant lymphoid cells. *Leuk. Lymphoma* **43**, 37–49.
- TANAKA, N., and TANIGUCHI, T. (2000). The interferon regulatory factors and oncogenesis. *Semin. Cancer Biol.* **10**, 73–81.
- THIRUKKUMARAN, C.M., LUDER, J.M., STEWART, D.A., CHENG, T., LUPICHUK, S.M., NODWELL, M.J., RUSSELL, J.A., AUER, I.A., and MORRIS, D.G. (2003). Reovirus oncolysis as a novel purging strategy for autologous stem cell transplantation. *Blood* **102**, 377–387.
- TSUBOI, K., IIDA, S., INAGAKI, H., KATO, M., HAYAMI, Y., HANAMURA, I., MIURA, K., HARADA, S., KIKUCHI, M., KOMATSU, H., BANNO, S., WAKITA, A., NAKAMURA, S., EMOTO, T., and UEDA, R. (2000). MUM1/IRF4 expression as a frequent event in mature lymphoid malignancies. *Leukemia* **14**, 449–456.
- TZOANPOULOS, D., SPELETAS, M., ARVANITIDIS, K., VEOPOULOU, C., KYRIAKI, S., THYPHRONITIS, G., SIDERAS, P., KARTALIS, G., and RITIS, K. (2002). Low expression of interferon regulatory factor-1 and identification of novel exons skipping in patients with chronic myeloid leukaemia. *Br. J. Haematol.* **119**, 46–53.

- VAN DEN BERGHE, H., VERMAELEN, K., MECUCCI, C., BARBIERI, D., and TRICOT, G. (1985). The 5q-anomaly. *Cancer Genet. Cytogenet.* **17**, 189–255.
- WILLMAN, C.L., SEVER, C.E., PALLAVICINI, M.G., HARADA, H., TANAKA, N., SLOVAK, M.L., YAMAMOTO, H., HARADA, K., MEEKER, T.C., LIST, A.F., *et al.* (1993). Deletion of IRF-1, mapping to chromosome 5q31.1, in human leukemia and preleukemic myelodysplasia. *Science* **259**, 968–971.
- YAMADA, M., ASANUMA, K., KOBAYASHI, D., MORI, R., YAJIMA, T., YAGIHASHI, A., YAMAMORI, S., and WATANABE, N. (2001). Quantitation of multiple myeloma oncogene 1/interferon-regulatory factor 4 gene expression in malignant B-cell proliferations and normal leukocytes. *Anticancer Res.* **21**, 633–638.
- YOSHIDA, S., NAKAZAWA, N., IIDA, S., HAYAMI, Y., SATO, S., WAKITA, A., SHIMIZU, S., TANIWAKI, M., and UEDA, R. (1999). Detection of MUM1/IRF4-IgH fusion in multiple myeloma. *Leukemia* **13**, 1812–1816.

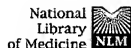
Address reprint requests to:

Dr. John Bell
Ottawa Regional Cancer Centre Research Laboratories
503 Smyth Road
Ottawa, Ontario, K1H 1C4
Canada

E-mail: jbell@ohri.ca

Received for publication January 29, 2004; accepted after revision July 6, 2004.

Published online: July 26, 2004.



All Databases PubMed Nucleotide Protein Genome Structure OMIM PMC Journals Book

Search PubMed for



Limits Preview/Index History Clipboard Details

Display Abstract Show 20 Sort by Send to

All: 1 Review: 0

[About Entrez](#)[Text Version](#)[Entrez PubMed](#)[Overview](#)[Help | FAQ](#)[Tutorial](#)[New/Noteworthy](#)[E-Utilities](#)[PubMed Services](#)[Journals Database](#)[MeSH Database](#)[Single Citation Matcher](#)[Batch Citation Matcher](#)[Clinical Queries](#)[Special Queries](#)[LinkOut](#)[My NCBI \(Cubby\)](#)[Related Resources](#)[Order Documents](#)[NLM Catalog](#)[NLM Gateway](#)[TOXNET](#)[Consumer Health](#)[Clinical Alerts](#)[ClinicalTrials.gov](#)[PubMed Central](#)☐ 1: J Gen Virol. 1980 Mar;47(1):89-96.[Related Articles, Links](#)

Interferon induction by viruses. III. Vesicular stomatitis virus: interferon-inducing particle activity requires partial transcription of gene N.

Marcus PI, Sekellick MJ.

We have measured the interferon-inducing particle (i.f.p.) activity of a ts mutant, GII (I), of vesicular stomatitis virus (VSV) and a non-ts revertant, RI (T1026) in "aged" chick embryo cells and mouse (L(Y) cells at 40.5 and 37.5 degrees C, respectively. Our results suggest that a single i.f.p. suffices to induce a quantum yield of interferon and that there are several times more i.f.p. than plaque-forming particles (p.f.p.) in stock preparations of VSV. Furthermore, while virus replication or amplified RNA synthesis is not required for a particle of VSV to induce interferon, there is a requirement for primary transcription. About one-tenth of the genome must remain intact and be transcribed to synthesize an interferon-inducer moiety. (This represents transcription of about two-thirds of the N protein gene.) We conclude that VSV does not contain a pre-formed inducer of interferon and propose a model for its formation. We suggest that there is a cumulative loss of N (and/or NS and L) protein from the ribonucleoprotein complex during primary transcription, leading ultimately to extensive base-pairing between the genome RNA and its complementary transcript. We suggest that the dsRNA thus formed constitutes the interferon inducer moiety of VSV.

PMID: 6154126 [PubMed - indexed for MEDLINE]

Display Abstract Show 20 Sort by Send to

[Write to the Help Desk](#)[NCBI | NLM | NIH](#)[Department of Health & Human Services](#)[Privacy Statement | Freedom of Information Act | Disclaimer](#)

Jun 27 2005 04:57:20



US005677178A

United States Patent [19]

McCormick

[11] Patent Number: 5,677,178
 [45] Date of Patent: Oct. 14, 1997

[54] CYTOPATHIC VIRUSES FOR THERAPY AND PROPHYLAXIS OF NEOPLASIA

[75] Inventor: Francis McCormick, Richmond, Calif.

[73] Assignee: ONYX Pharmaceuticals, Inc.,
 Richmond, Calif.

[21] Appl. No.: 641,081

[22] Filed: Apr. 29, 1996

Related U.S. Application Data

[63] Continuation of Ser. No. 198,184, Feb. 16, 1994, abandoned, which is a continuation-in-part of Ser. No. 17,525, Feb. 16, 1993, abandoned.

[51] Int. Cl.⁶ C12N 5/10; C12N 7/01;
 C07H 21/04

[52] U.S. Cl. 435/325; 435/235.1; 435/375;
 536/23.1; 536/24.1

[58] Field of Search 435/240.2, 325,
 435/375, 235.1, 101; 536/23.1, 24.1

[56] References Cited

FOREIGN PATENT DOCUMENTS

91308439 9/1991 European Pat. Off.
 WO 93/19191 9/1993 WIPO
 PCT/US95/
 07858 6/1995 WIPO

OTHER PUBLICATIONS

Shingu et al (1991) J Ger Virol 72 2031-2034.
 Matuza et al (1991) Science 252: 854-856.
 Horwitz 1985 Virology Chapter 24 477-495.

Whyte et al (1989) Cell vol. 56 67-75.

Burk & Barker 1987 Virology 156:107-121.

Smith et al., Studies on the Use of Viruses in the Treatment of Carcinoma of the Cervix, *Cancer*, vol. 9, pp. 1211-1218 (1956).

Newman et al., Virus Treatment in Advanced Cancer, *Cancer*, vol. 7, pp. 106-118, (1954).

Alice E. Moore. Effects of Viruses on Tumors, *Ann Rev Microbiol*, vol. 8, pp. 393-410 (1954).

F. L. Graham, Transformation by and Oncogenicity of Human Adenoviruses, *The Adenoviruses*, Chapter 9, pp. 339-383 (1984).

Bjorn et al., Selective Elimination of Breast Cancer Cells from Human Bone Marrow Using an Antibody-Pseudomonas, *Cancer Research*, vol. 50, pp. 5992-5996, (1990).

Primary Examiner—Nancy Degen

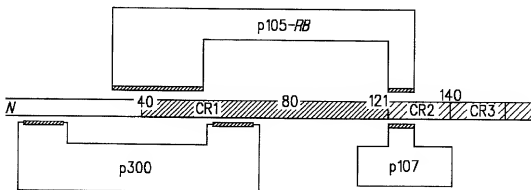
Attorney, Agent, or Firm—Gregory Giotta; Timothy E. Torchia

[57]

ABSTRACT

Methods and compositions for treating neoplastic conditions by viral-based therapy are provided. Mutant virus lacking viral proteins which bind and/or inactivate p53 or RB are administered to a patient having a neoplasm which comprises cells lacking p53 and/or RB function. The mutant virus is able to substantially produce a replication phenotype in neoplastic cells but is substantially unable to produce a replication phenotype in non-replicating, non-neoplastic cells having essentially normal p53 and/or RB function. The preferential generation of replication phenotype in neoplastic cells results in a preferential killing of the neoplastic cells, either directly or by expression of a cytotoxic gene in cells expressing a viral replication phenotype.

29 Claims, 9 Drawing Sheets



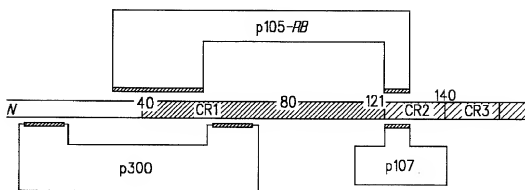


FIGURE 1

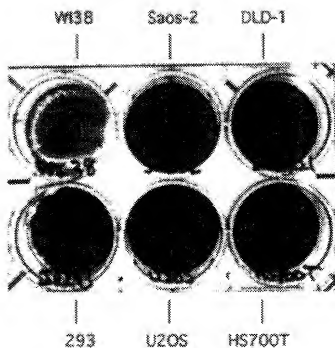


FIGURE 2A

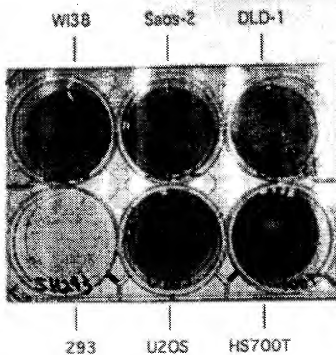


FIGURE 2B

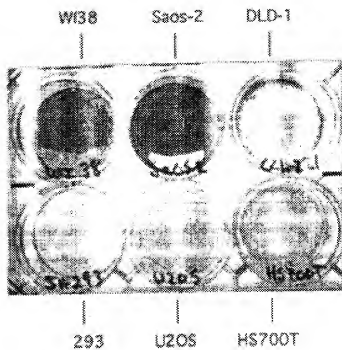


FIGURE 2C

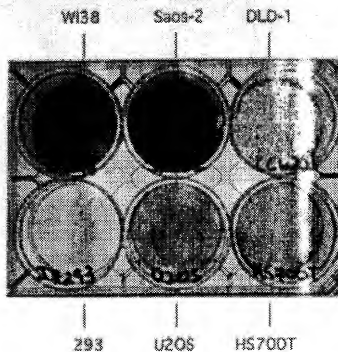


FIGURE 2D

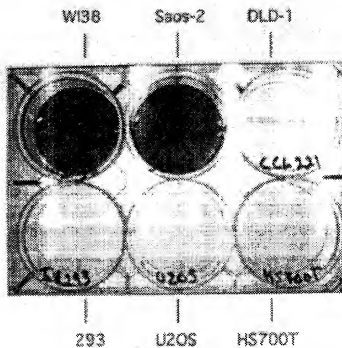


FIGURE 2E

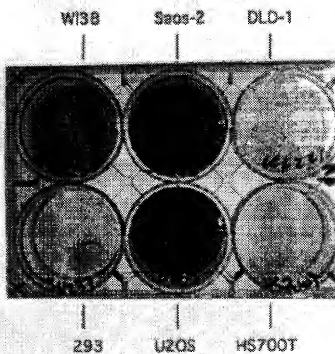


FIGURE 2F

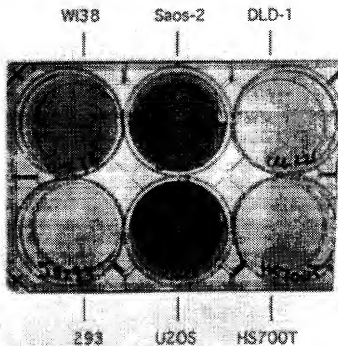


FIGURE 2G

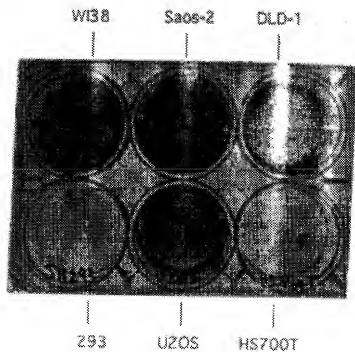


FIGURE 2H

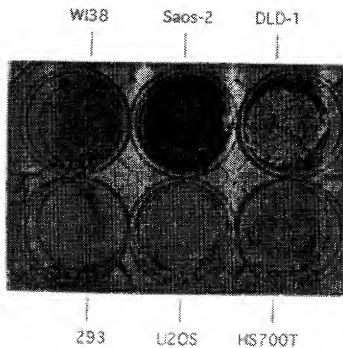


FIGURE 2I

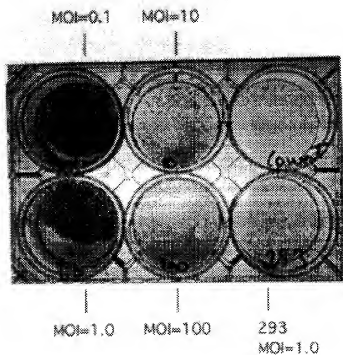


FIGURE 3A

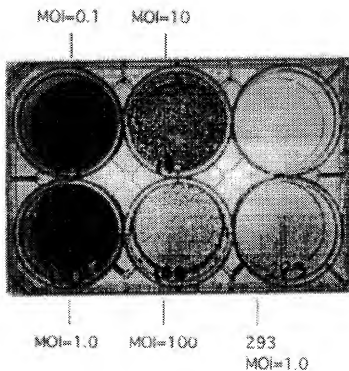


FIGURE 3B

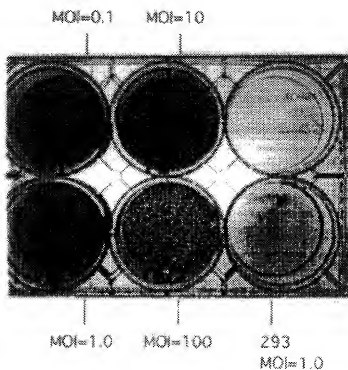


FIGURE 3C

CYTOPATHIC VIRUSES FOR THERAPY AND PROPHYLAXIS OF NEOPLASIA

This application is a continuation of U.S. Ser. No. 08/198,184, filed Feb. 16, 1994, now abandoned, which is a continuation-in-part of U.S. Ser. No. 08/017,525, filed Feb. 16, 1993 now abandoned.

The invention provides compositions of recombinant cytopathic viruses which are capable of replication and/or expression of late region genes in neoplastic mammalian cells but are essentially non-replicable in non-neoplastic cells, methods for constructing and propagating such recombinant viruses, methods for treating neoplastic disease with such recombinant viruses, and therapeutic compositions comprising such recombinant viruses.

BACKGROUND

The proliferation of normal cells is thought to be regulated by growth-promoting proto-oncogenes counterbalanced by growth-constraining tumor-suppressor genes. Mutations that potentiate the activities of proto-oncogenes create the oncogenes that force the growth of neoplastic cells. Conversely, genetic lesions that inactivate tumor suppressor genes, generally through mutation(s) that lead to a cell being homozygous for the inactivated tumor suppressor allele, can liberate the cell from the normal replicative constraints imposed by these genes. Usually, an inactivated tumor suppressor gene (e.g., p53, RB, DCC, NF-1) in combination with the formation of an activated oncogene (i.e., a proto-oncogene containing an activating structural or regulatory mutation) can yield a neoplastic cell capable of essentially unconstrained growth (i.e., a transformed cell).

Oncogenic transformation of cells leads to a number of changes in cellular metabolism, physiology, and morphology. One characteristic alteration of oncogenically transformed cells is a loss of responsiveness to constraints on cell proliferation and differentiation normally imposed by the appropriate expression of cell-growth regulatory genes.

While different types of genetic alterations may all lead to altered expression or function of cell-growth regulatory genes and to abnormal growth, it is generally believed that more than one event is required to lead to neoplastic transformation of a normal cell to a malignant one (Land et al. (1983) *Nature* 304: 596; Weinberg, R. A. (1989) *Cancer Res.* 49: 3713). The precise molecular pathways and secondary changes leading to malignant transformation for most cell types are not clear. A number of cases have been reported in which altered expression or activity of some proteins with putative cell-cycle control functions and/or implicated in the formation of functional transcriptional complexes, such as p53 and RB, can lead to loss of proliferation control in cells (Ullrich et al. (1992) *J. Biol. Chem.* 267: 15259; Hollstein et al. (1991) *Science* 253: 49; Sager, R. (1992) *Curr. Opin. Cell. Biol.* 4: 155; Levine et al. (1991) *Nature* 351: 453).

Some oncogenes have been found to possess characteristic activating mutations in a significant fraction of certain cancers. For example, particular mutations in the *ras*^H and *ras*^K coding regions (e.g., codon 12, codon 61; Parada et al. (1984) *Nature* 312: 649) and the APC gene (Powell et al. (1992) *Nature* 359: 235) are associated with oncogenic transformation of cultured cells and are present in a striking percentage of specific human cancers (e.g., colon adenocarcinoma, bladder carcinoma, lung carcinoma and adenocarcinoma, hepatocarcinoma). These findings have led to the development of diagnostic and therapeutic reagents

(e.g., polynucleotide probes and antibodies) that specifically recognize the activated form(s) of such oncogenes (U.S. Pat. No. 4,798,787 and U.S. Pat. No. 4,762,706).

The excessive or inappropriate expression of other oncogenes, such as *myc*, *erbB-2*, and *plm-1*, appears to be able to potentiate oncogenic transformation without necessarily requiring the presence of activating mutation(s) in the coding region. Overexpression of *erbB-2* is frequently found in adenocarcinoma of the breast, stomach, and ovary, and *erbB-2* levels in these cell types might serve as a diagnostic marker for neoplasia and/or may correlate with a specific tumor phenotype (e.g., resistance to specific drugs, growth rate, differentiation state).

Transgenic animals harboring various oncogenes (U.S. Pat. No. 4,736,866 and U.S. Pat. No. 5,087,571) or functionally disrupted tumor suppressor genes (Donehower et al. (1992) *Nature* 356: 215) have been described for use in carcinogen screening assays, among other potential uses.

Despite this progress in developing a more defined model of the molecular mechanisms underlying the transformed phenotype and neoplasia, few significant therapeutic methods applicable to treating cancer beyond conventional chemotherapy have resulted. Many conventional chemotherapeutic agents have a low therapeutic index, with therapeutic dosage levels being at or near dosage levels which produce toxicity. Toxic side effects of most conventional chemotherapeutic agents are unpleasant and lead to life-threatening bone marrow suppression, among other side effects.

Recent approaches for performing gene therapy to correct or supplement defective alleles which cause congenital diseases, such as cystic fibrosis, have been attempted with reports of limited initial success. Some gene therapy approaches involve transducing a polynucleotide sequence capable of expressing a functional copy of a defective allele into a cell in vivo using replication-deficient recombinant adenovirus (Rosenfeld et al. (1992) *Cell* 68: 143). Some of these gene therapy methods are efficient at transducing polynucleotides into isolated cells explanted from a patient, but have not been shown to be highly efficient in vivo. Therapeutic approaches to cancer which rely on transfection of explanted tumor cells with polynucleotides encoding tumor necrosis factor (TNF) and interleukin-2 (IL-2) have been described (Pardoll, D. (1992) *Curr. Opin. Oncol.* 4: 1124).

Although it might someday prove possible for gene therapy methods to be adapted to correct defective alleles of oncogenes or tumor suppressor genes in transformed cells in vivo, present gene therapy methods have not been reported to be able to efficiently transduce and correctly target (e.g., by homologous recombination) a sufficient percentage of neoplastic cells for practical gene therapy of neoplasia in situ. The nature of cancer biology mandates that a substantial fraction of the neoplastic cells, preferably all of the clonal progeny of the transformed cell, are ablated for an effective therapeutic effect. Moreover, present methods for gene therapy are very expensive, requiring ex vivo culturing of explanted cells prior to reintroduction into a patient. Widespread application of such methods, even if they were effective, would be prohibitively expensive.

Thus there exists a need in the art for methods and compositions for diagnosis and therapy of neoplastic diseases, especially for methods which selectively ablate neoplastic cells without the undesirable killing of non-neoplastic cells that is typical of conventional antineoplastic chemotherapy. The present invention fulfills these and other needs.

The references discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention.

SUMMARY OF THE INVENTION

The present invention provides several novel methods and compositions for ablating neoplastic cells by infecting the neoplastic cells with a recombinant adenovirus which is substantially replication deficient in non-neoplastic cells and which exhibits at least a partial replication phenotype in neoplastic cells. The difference in replication phenotype of the adenovirus constructs of the invention in neoplastic and non-neoplastic cells provides a biological basis for viral-based therapy of cancer. Expression of adenoviral cytopathic effects, and optionally expression of a negative-selectable drug gene (e.g., HSV tk), are correlated with the adenoviral replication phenotype characteristic of neoplastic cells infected with the recombinant adenovirus constructs of the invention, thus discriminating between neoplastic and non-neoplastic cells and providing selective cytotoxicity of neoplastic cells. Although the methods are described in detail specifically for adenoviral constructs, the methods are believed to be applicable to essentially any virus type wherein efficient replication requires binding and/or sequestration and/or inactivation of a host cell protein that is present in non-neoplastic cells but is substantially absent or nonfunctional in neoplastic cells (e.g., p53, RB).

In order for adenovirus to replicate efficiently in cells, the adenoviral E1b gene product, p55, forms a complex with the host cell p53 protein, thereby sequestering and/or inactivating p53 and producing a cell that is deficient in p53 function. Such a cell made deficient in p53 function can support replication of the adenovirus. In this way, wild-type adenovirus is able to replicate in cells containing p53, as the adenovirus p55 proteins inactivates and/or sequesters the host cell p53 protein. In one embodiment of the invention, a recombinant adenovirus comprising an E1b locus encoding a mutant p55 protein that is substantially incapable of forming a functional complex with p53 protein in infected cells is administered to an individual or cell population comprising a neoplastic cell capable of being infected by the recombinant adenovirus. The substantial incapacity of the recombinant adenovirus to effectively sequester p53 protein in infected non-neoplastic cells results in the introduced recombinant adenoviral polynucleotide(s) failing to express a replication phenotype in non-neoplastic cells. By contrast, neoplastic cells which lack a functional p53 protein support expression of a replication phenotype by the introduced recombinant adenovirus which leads to ablation of the neoplastic cell by an adenoviral cytopathic effect and/or expression of a negative selection gene linked to the replication phenotype. In preferred variations of these embodiments, the recombinant adenovirus comprises an E1b locus encoding a mutant p55 which is substantially incapable of binding p53 and may optionally also lack a functional p19 protein (i.e., incapable of inhibiting expression of adenoviral early region genes in the presence of E1a polypeptides). Recombinant adenoviruses of the invention may further comprise a mutant p19 gene which produces enhanced cytopathic effects; such a mutant known in the art is the p19 cyt mutant gene. However, it may be preferable to retain functional p19 in some mutants to maintain the integrity of viral DNA during the infection.

In an alternative embodiment of the invention, a recombinant adenovirus comprising an E1a locus encoding an E1a

protein (e.g., p289R or p243R) that is substantially incapable of forming a complex with RB protein in infected cells is administered to an individual or cell population comprising a neoplastic cell capable of being infected by the recombinant adenovirus. The substantial incapacity of the recombinant adenovirus to effectively sequester RB protein in infected non-neoplastic cells results in the introduced recombinant adenoviral polynucleotide(s) failing to express a replication phenotype in non-neoplastic cells. By contrast, neoplastic cells which lack a functional RB protein support expression of a replication phenotype by the introduced recombinant adenovirus which leads to ablation of the neoplastic cell by an adenoviral cytopathic effect and/or expression of a negative selection gene linked to the replication phenotype. In preferred variations of these embodiments, the recombinant adenovirus comprises an E1a locus encoding a mutant E1a protein (e.g., p289R) that lacks a CR1 and/or CR2 domain capable of binding RB (and/or the 300 kD polypeptide and/or the 107 kD polypeptide) but comprises a functional CR3 domain capable of transactivation of adenoviral early genes. Additional variations of these embodiments include those where the recombinant adenovirus comprises a nonfunctional E1a locus which is substantially incapable of expressing a protein that binds to and inactivates RB and may optionally also comprise a functional p19 protein (i.e., capable of stimulating expression of adenoviral early region genes in the absence of E1a function). Recombinant adenoviruses of the invention may further comprise a mutant p19 gene which produces enhanced cytopathic effects; such a mutant known in the art is the p19 cyt mutant gene.

The invention provides novel recombinant adenovirus constructs which are replication defective in non-neoplastic cells but capable of expressing a replication phenotype in neoplastic cells lacking functional p53 and/or RB. The novel recombinant adenovirus constructs comprise a mutation, such as a deletion or point mutation, in the E1a and/or E1b gene regions, especially in the sequences encoding the E1b p55 protein and the CR1 and CR2 domains of the E1a p289R or p243R proteins. In some embodiments, a negative selectable gene, such as an HSV tk gene, is operably linked to an early region (e.g., E2, E1a, E1b) enhancer/promoter, a late region gene enhancer/promoter (e.g., major late promoter), or an early or late region promoter with a CMV enhancer, in a recombinant adenovirus construct also comprising an E1a or E1b mutation, so that the negative selectable gene is preferentially transcribed in infected cells which express a replication phenotype (i.e., neoplastic cells) and provides negative selection of such cells by administration of an effective dosage of a negative selection agent (e.g., gancyclovir, FIAU). A negative selectable gene may be inserted in place of an E1a and/or E1b structural sequence to concomitantly form an E1a⁻ replication deficient mutant, E1b⁻ replication deficient mutant, or E1a/E1b double mutant, respectively.

Antineoplastic compositions comprising such recombinant adenovirus in a pharmaceutically acceptable form for delivery to a neoplastic cell population in vivo are also provided.

The invention also provides recombinant papovaviruses, such as human papillomavirus (HPV), polyomaviruses (e.g., BK, JC) and SV40, which lack functional proteins for binding and/or inactivating p53 and/or RB. Human papillomavirus mutants lacking expression of functional E6 protein will substantially lack the capacity to effectively degrade p53 and thus will be capable of manifesting a replication phenotype in p53⁻ cells but not in cells containing a

sufficient level of functional p53. Human papillomavirus mutants lacking expression of functional E7 protein will substantially lack the capacity to effectively bind RB and thus will be capable of manifesting a replication phenotype in RB⁽⁻⁾ cells but not in cells containing a sufficient level of functional RB. Human papillomavirus mutants lacking expression of both functional E6 protein and functional E7 protein will substantially lack the capacity to effectively bind RB and p53 thus will be capable of manifesting a replication phenotype in p53⁽⁻⁾RB⁽⁻⁾ cells but not in cells containing a sufficient level of functional RB and/or p53.

The invention also provides novel methods for treating a neoplastic disease comprising the steps of administering to a patient a recombinant virus capable of preferentially expressing a replication phenotype and/or expressing a cytopathic effect in a neoplastic cell population as compared to expression in a non-neoplastic cell population.

BRIEF DESCRIPTION OF THE DRAWING

FIG. 1 shows a schematic representation of the domain structure and protein-protein interactions of an adenovirus E1a-289R polypeptide.

FIGS. 2(a-i) shows infection of normal and neoplastically transformed human cell lines with wild-type adenovirus and recombination-deficient recombinant adenovirus mutants. Each cell line indicated was mock-infected, FIG. 2(a); or infected at an MOI of 1.0 with wild-type adenovirus 2, FIGS. 2(b-c); mutant derivative dl 1010, FIGS. 2(d-e); mutant derivative dl 1520, FIGS. 2(f-g); or mutant derivative dl 434, FIGS. 2(h-i). FIG. 2(a) shows results 9 days after the mock infection. The remaining panels show results from the following post-infection periods: FIG. 2(b), 4 days; FIG. 2(c), 7 days; FIG. 2(d), 8 days; FIG. 2(e), 12 days; FIG. 2(f), 14 days; FIG. 2(g), 20 days; FIG. 2(h), 6 days; FIG. 2(i), 14 days. On various days post-infection (noted supra), the cells were washed with PBS to remove dead cells and the remaining cells were stained with crystal violet. Each cell line mock infected with PBS alone was included as a control for viability.

FIGS. 3(a-c) shows infection of the normal human lung diploid fibroblast cell line IMR90 (ATCC CCL 186) with wild-type and mutant adenoviruses. IMR90 cells were infected with the indicated virus at MOIs corresponding to 0.1, 1.0, 10, and 100. At the indicated days post-infection, cells were washed with PBS and stained with crystal violet. One well in each 6-well dish was seeded with 293 cells to serve as a positive control for virus infection. FIG. 3(a) shows cells infected with wild-type Ad2 at 9 days post-infection. FIG. 3(b) shows cells infected with dl 1010 at 9 days post-infection. FIG. 3(c) shows cells infected with dl 1520 at 9 days post-infection.

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described. For purposes of the present invention, the following terms are defined below.

The term "naturally-occurring" as used herein as applied to an object refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses)

that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally-occurring. As used herein, the term "recombinant" indicates that a polynucleotide construct (e.g., and adenovirus genome) has been generated, in part, by intentional modification by man.

As used herein, the term "replication deficient virus" refers to a virus that preferentially inhibits cell proliferation or induces apoptosis in a predetermined cell population (e.g., cells substantially lacking p53 and/or RB function) which supports expression of a virus replication phenotype, and which is substantially unable to inhibit cell proliferation, induce apoptosis, or express a replication phenotype in cells comprising normal p53 and RB function levels characteristic of non-replicating, non-transformed cells. Typically, a replication deficient virus exhibits a substantial decrease in plaquing efficiency on cells comprising normal RB and/or p53 function.

As used herein, the term "p53 function" refers to the property of having an essentially normal level of a polypeptide encoded by the p53 gene (i.e., relative to non-neoplastic cells of the same histological type), wherein the p53 polypeptide is capable of binding an E1b p55 protein of wild-type adenovirus 2 or 5. For example, p53 function may be lost by production of an inactive (i.e., mutant) form of p53 or by a substantial decrease or total loss of expression of p53 polypeptide(s). Also, p53 function may be substantially absent in neoplastic cells which comprise p53 alleles encoding wild-type p53 protein; for example, a genetic alteration outside of the p53 locus, such as a mutation that results in aberrant subcellular processing or localization of p53 (e.g., a mutation resulting in localization of p53 predominantly in the cytoplasm rather than the nucleus) can result in a loss of p53 function.

As used herein, the term "RB function" refers to the property of having an essentially normal level of a polypeptide encoded by the RB gene (i.e., relative to non-neoplastic cells of the same histological type), wherein the RB polypeptide is capable of binding an E1a protein of wild-type adenovirus 2 or 5. For example, RB function may be lost by production of an inactive (i.e., mutant) form of RB or by a substantial decrease or total loss of expression of RB polypeptide(s). Also, RB function may be substantially absent in neoplastic cells that comprise RB alleles encoding a wild-type RB protein; for example, a genetic alteration outside of the RB locus, such as a mutation that results in aberrant subcellular processing or localization of RB, may result in a loss of RB function.

As used herein, the term "replication phenotype" refers to one or more of the following phenotypic characteristics of cells infected with a virus such as a replication deficient adenovirus: (1) substantial expression of late gene products, such as capsid proteins (e.g., adenoviral penton base polypeptide) or RNA transcripts initiated from viral late gene promoter(s), (2) replication of viral genomes or formation of replicative intermediates, (3) assembly of viral capsids or packaged virion particles, (4) appearance of cytopathic effect (CPE) in the infected cell, (5) completion of a viral lytic cycle, and (6) other phenotypic alterations which are typically contingent upon abrogation of p53 or RB function in non-neoplastic cells infected with a wild-type replication competent DNA virus encoding functional oncoprotein(s). A replication phenotype comprises at least one of the listed phenotypic characteristics, preferably more than one of the phenotypic characteristics.

The term "antineoplastic replication deficient virus" is used herein to refer to a recombinant virus which has the

functional property of inhibiting development or progression of a neoplasm in a human, by preferential cell killing of infected neoplastic cells relative to infected nonreplicating, non-neoplastic cells of the same histological cell type.

As used herein, "neoplastic cells" and "neoplasia" refer to cells which exhibit relatively autonomous growth, so that they exhibit an aberrant growth phenotype characterized by a significant loss of control of cell proliferation. Neoplastic cells comprise cells which may be actively replicating or in a temporary non-replicative resting state (G_1 or G_0); similarly, neoplastic cells may comprise cells which have a well-differentiated phenotype, a poorly-differentiated phenotype, or a mixture of both type of cells. Thus, not all neoplastic cells are necessarily replicating cells at a given timepoint. The set defined as neoplastic cells consists of cells in benign neoplasms and cells in malignant (or frank) neoplasms. Frankly neoplastic cells are frequently referred to as cancer, typically termed carcinoma if originating from cells of endodermal or ectodermal histological origin, or sarcoma if originating from cell types derived from mesoderm.

As used herein, the term "operably linked" refers to a linkage of polynucleotide elements in a functional relationship. A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the coding sequence. Operably linked means that the DNA sequences being linked are typically contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame. However, since enhancers generally function when separated from the promoter by several kilobases and intronic sequences may be of variable lengths, some polynucleotide elements may be operably linked but not contiguous.

As used herein, "physiological conditions" refers to an aqueous environment having an ionic strength, pH, and temperature substantially similar to conditions in an intact mammalian cell or in a tissue space or organ of a living mammal. Typically, physiological conditions comprise an aqueous solution having about 150 mM NaCl (or optionally KCl), pH 6.5–8.1, and a temperature of approximately 20°–45° C. Generally, physiological conditions are suitable binding conditions for intermolecular association of biological macromolecules. For example, physiological conditions of 150 mM NaCl, pH 7.4, at 37° C. are generally suitable.

DETAILED DESCRIPTION

Generally, the nomenclature used hereafter and the laboratory procedures in cell culture, molecular genetics, and molecular virology described below are those well known and commonly employed in the art. Standard techniques are used for recombinant nucleic acid methods, polynucleotide synthesis, polypeptide synthesis, generation and propagation of virus stocks (including cell lines capable of transcomplementation of replication deficient virus stocks), cell culture, and the like. Generally enzymatic reactions and purification steps are performed according to the manufacturer's specifications. The techniques and procedures are generally performed according to conventional methods in the art and various general references (see, generally, Sambrook et al. *Molecular Cloning: A Laboratory Manual*, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.; *Virology*, Second edition, eds. Fields, B. N. and Knipe, D. M., (1990) Raven Press, New York, N.Y., incorporated herein by reference) which are provided throughout this

document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

Neoplasia is a pathological condition which is characterized, in part, by the generation of neoplastic cells having variant genotypes and phenotypes. Some tumors may comprise a population of cells lacking RB function but having p53 function; such cells are designated $RB^{-/-}$. Some tumor cells may lack p53 function but have RB function; such cells are designated $p53^{-/-}$. Some tumors may comprise cells lacking both p53 and RB and are designated $p53^{-/-}RB^{-/-}$. The cell line SAOS2 (infra, see Experimental Example) is an example of a neoplastic cell type which is $p53^{-/-}RB^{-/-}$. Also, there may be neoplastic cells which comprise essentially normal levels of p53 and RB; such cells having both normal p53 and normal RB may lack other oncoproteins (e.g., tumor suppressor gene products other than p53 or RB) which can provide the basis for antineoplastic viral constructs which can preferentially manifest a replication phenotype in such neoplastic cells.

A basis of the present invention is that several DNA viruses which infect mammalian cells (e.g., adenoviruses; papovaviruses such as BK and JC, SV40, and papillomaviruses such as HPV, and the like) encode viral proteins which are essential for efficient progression through the viral replication cycle; some of these viral proteins sequester cellular proteins, such as those involved in cell-cycle control and/or formation of transcription complexes, as a necessary condition for efficient viral replication. In the absence of the viral proteins which bind, sequester, or degrade such cellular proteins as p53 and RB, viral replication is substantially inhibited. Normal (i.e., non-neoplastic) cells which are infected with a mutant virus lacking the ability to sequester or degrade p53 and/or RB are generally unable to support replication of the mutant virus, hence such mutant viruses are considered to be replication deficient (or replication defective). However, since the sequestration or degradation of p53 or RB is not necessary for viral replication in cells which lack functional p53 or RB (such cells are designated $p53^{-/-}$ and $RB^{-/-}$ respectively) it is possible that replication deficient mutant viruses which are defective for p53 and/or RB sequestration or degradation may express a replication phenotype in such $p53^{-/-}$ or $RB^{-/-}$ cells to a greater extent than in cells having essentially normal p53 and/or RB function. Neoplastic cells frequently lack p53 function (a $p53^{-/-}$ cell), RB function (a $RB^{-/-}$ cell), or both functions (a $p53^{-/-}RB^{-/-}$ cell). Hence, some replication deficient viral mutants may preferentially exhibit a replication phenotype in neoplastic cells.

Viral mutants lacking the capacity to express a functional RB inactivating protein (e.g., adenovirus E1a, HPV E7 protein) will manifest a replication phenotype in $RB^{-/-}$ cells and $RB^{-/-}p53^{-/-}$ cells. Viral mutants lacking the capacity to express a functional p53 inactivating protein (e.g., adenovirus E1b p55, HPV E6 protein) will manifest a replication phenotype in $p53^{-/-}$ cells and $RB^{-/-}p53^{-/-}$ cells. Viral mutants lacking the capacity to express both a functional p53 inactivating protein (e.g., adenovirus E1b p55, HPV E6 protein) and a functional RB inactivating protein (e.g., adenovirus E1a, HPV E7 protein) will manifest a replication phenotype in $RB^{-/-}p53^{-/-}$ cells. Cytotoxicity linked to the expression of a replicative phenotype can therefore be used as a basis for preferentially killing neoplastic cells having a $RB^{-/-}$, $p53^{-/-}$, or $RB^{-/-}p53^{-/-}$ phenotype. Although some replicating non-neoplastic cells may transiently exhibit a $RB^{-/-}$ phenotype, $p53^{-/-}$ phenotype, or $RB^{-/-}p53^{-/-}$ pheno-

type during progression through the cell cycle, the viral mutants of the invention may be used for preferential, albeit not necessarily completely selective, killing of neoplastic cells, thus constituting a useful antineoplastic therapy modality to be used alone or in combination with other modalities of treatment. Deletions (or other inactivating mutations) in the 37 amino-terminal residues of the HPV E7 polypeptide are preferred HPV mutants for application to RB^{-/-} cells, since these residues are important for RB binding.

Although the methods and compositions presented below are described specifically for methods relating to replication deficient adenoviral constructs, it is believed that the invention can be practiced with other DNA viruses encoding oncoproteins which sequester or enhance the degradation of p53 protein or RB protein, for example replication deficient papillomavirus species (e.g., mutants of HPV types 16, 18, 33) that contain mutations in the E6 and/or E7 genes which substantially abrogate p53 and/or RB function, respectively. In addition to members of the family Adenoviridae (specifically the genus Mastadenovirus), it is believed that members of the family Papovaviridae, especially papillomavirus and polyomavirus, which encode viral proteins that sequester and/or inactivate p53 or RB are suitable for use in the methods of the invention.

For a general description of adenovirus and papovavirus biology, *Virology*, Second edition, eds. Fields, B. N. and Knipe, D. M., Vol. 2, pp. 1651-1740, Raven Press, New York, N.Y., incorporated herein by reference, may be referred to for guidance. The following specific descriptions refer to, but are not limited to, adenovirus serotype 5 and adenovirus serotype 2. Although it is believed that other adenovirus serotypes may be used, adenovirus type 5 provides a common reference point for the nucleotide numbering convention of viral polynucleotides and amino acid numbering of viral-encoded polypeptides of the E1a viral gene region, and other viral genes. Adenovirus type 2 provides a convenient reference for the numbering convention of the E1b viral gene region, and other viral gene regions. It is believed that those of skill in the art will readily identify the corresponding positions in other adenovirus serotypes. References to human papillomavirus generally refer to a type associated with neoplasia (e.g., types 16, 18, or 33), although non-oncogenic types may also be used.

E1a Mutants

The loss of retinoblastoma tumor suppressor gene (RB) gene function has been associated with the etiology of various types of tumors. The product of this tumor suppressor gene, a 105 kilodalton polypeptide called pRB or p105, is a cell-cycle regulatory protein. The pRB polypeptide inhibits cell proliferation by arresting cells at the G₁ phase of the cell cycle. The pRB protein is also a major target of several DNA virus oncoproteins, including adenovirus E1a, SV40 large T Ag, and papillomavirus E7. These viral proteins bind and inactivate pRB, and the function of inactivating pRB is important in facilitating viral replication. The pRB protein interacts with the E2F transcription factor, which is involved in the expression of the adenovirus E2 gene and several cellular genes, and inhibits the activity of this transcription factor (Bagchi et al. (1991) *Cell* 65: 1063; Bandaru et al. (1991) *Nature* 351: 494; Chellappan et al. (1992) *Proc. Natl. Acad. Sci. (U.S.A.)* 89: 4549, incorporated herein by reference). The viral oncoproteins, such as adenovirus E1a, disrupt the pRB/E2F complex resulting in activation of E2F. However, cells lacking sufficient functional pRB to complex the E2F will not require the presence of a

functional oncoprotein, such as E1a, to possess transcriptionally active E2F. Therefore, it is believed that replication deficient adenovirus species which lack the capacity to complex RB but substantially retain other essential replicative functions will exhibit a replication phenotype in cells which are deficient in RB function (e.g., cells which are homozygous or heterozygous for substantially deleted RB alleles, cells which comprise RB alleles encoding mutant RB proteins which are essentially nonfunctional, cells which comprise mutations that result in a lack of function of an RB protein) but will not substantially exhibit a replicative phenotype in non-replicating, non-neoplastic cells. Such replication deficient adenovirus species are referred to herein for convenience as E1a-RB^{-/-} replication deficient adenoviruses.

A cell population (such as a mixed cell culture or a human cancer patient) which comprises a subpopulation of neoplastic cells lacking RB function and a subpopulation of non-neoplastic cells which express essentially normal RB function can be contacted under infective conditions (i.e., conditions suitable for adenoviral infection of the cell population, typically physiological conditions) with a composition comprising an infectious dosage of a E1a-RB^{-/-} replication deficient adenovirus. Such contacting results in infection of the cell population with the E1a-RB^{-/-} replication deficient adenovirus. The infection produces preferential expression of a replication phenotype in a significant fraction of the cells comprising the subpopulation of neoplastic cells lacking RB function but does not produce a substantial expression of a replicative phenotype in the subpopulation of non-neoplastic cells having essentially normal RB function. The expression of a replication phenotype in an infected RB^{-/-} cell results in the death of the cell, such as by cytopathic effect (CPE), cell lysis, apoptosis, and the like, resulting in a selective ablation of neoplastic RB^{-/-} cells from the cell population.

Typically, E1a-RB^{-/-} replication deficient adenovirus constructs suitable for selective killing of RB^{-/-} neoplastic cells comprise mutations (e.g., deletions, substitutions, frameshifts) which inactivate the ability of an E1a polypeptide to bind RB protein effectively. Such inactivating mutations typically occur in the E1a CR1 domain (amino acids 30-85 in Ad5; nucleotide positions 697-790) and/or the CR2 domain (amino acids 120-139 in Ad5; nucleotide positions 920-967), which are involved in binding the p105 RB protein and the p107 protein. Preferably, the CR3 domain (spanning amino acids 150-186) remains and is expressed as a truncated p289R polypeptide and is functional in transactivation of adenoviral early genes. FIG. 1 portrays schematically the domain structure of the E1a-289R polypeptide.

Suitable E1a-RB^{-/-} replication deficient adenovirus constructs for use in the methods and compositions of the invention include, but are not limited to the following examples: (1) adenovirus serotype 5 NT dl 1010, which encodes an E1a protein lacking the CR1 and CR2 domains (deletion of amino acids 2 to 150; nucleotides 560-1009) necessary for efficient RB binding, but substantially retaining the CR3 domain (Whyte et al. (1989) *Cell* 56: 67, incorporated herein by reference), and (2) adenovirus serotype 5 dl 312, which comprises a deleted viral genome lacking the region spanning nucleotides 448-1349 which encodes the entire E1a region in wild-type adenovirus (Jones, N. and Shenk, T. (1979) *Proc. Natl. Acad. Sci. (U.S.A.)* 76: 3665, incorporated herein by reference). Ad5 NT dl 1010 is a preferred E1a-RB^{-/-} replication deficient adenovirus and is available from Dr. E. Harlow, Massachusetts General Hospital, Boston, Mass.).

It may be preferable to incorporate additional mutations into such adenovirus constructs to inhibit formation of infectious virions in neoplastic cells which otherwise would support replication of the E1a-RB^{-/-} mutants. Such additional inactivating mutations would be preferred in therapeutic modalities wherein complete viral replication forming infectious virions capable of spreading to and infecting adjacent cells is undesirable. These fully inactivated mutants are referred to as nonreplicable E1a-RB^{-/-} mutants. Such nonreplicable mutants comprise mutations which prevent formation of infectious virions even in p53^{-/-}RB^{-/-} cells; such mutations typically are structural mutations in an essential virion protein or protease.

However, in many modalities it is desirable for the mutant virus to be replicable and to form infectious virions containing the mutant viral genome which may spread and infect other cells, thus amplifying the antineoplastic action of an initial dosage of mutant virus.

Additional E1a^{-/-} mutants lacking the capacity to bind RB can be generated by those of skill in the art by generating mutations in the E1a gene region encoding E1a polypeptides, typically in the CR1 and/or CR2 domains, expressing the mutant E1a polypeptide, contacting the mutant E1a polypeptides with p105 or a binding fragment of RB under aqueous binding conditions, and identifying mutant E1a polypeptides which do not specifically bind RB as being candidate E1a^{-/-} mutants suitable for use in the invention. Alternative assays include contacting the mutant E1a polypeptides with the 300 kD protein and/or p107 protein or binding fragment thereof under aqueous binding conditions, and identifying mutant E1a polypeptides which do not specifically bind the 300 kD and/or p107 polypeptides as being candidate E1a^{-/-} mutants suitable for use in the invention. Alternative binding assays include determining the inability of E1a^{-/-} mutant protein (or absence of E1a protein) to form complexes with the transcription factor E2F and/or to lack the ability to dissociate the RB protein from RB:E2F complexes under physiological conditions (Chellappan et al. (1991) op. cit., incorporated herein by reference).

Alternative functional assays for determining mutants lacking E1a function, such as loss of transactivation by E1a of transcription of various reporter polypeptides linked to a E1a-dependent transcriptional regulatory sequence, and the like, will be used.

E1b Mutants

A function of the cellular phosphoprotein p53 is to inhibit the progression of mammalian cells through the cell cycle. Wild-type adenovirus E1b p55 protein binds to p53 in infected cells that have p53 and produce a substantial inactivation of p53 function, likely by sequestering p53 in an inactive form. Functional E1b p55 protein is essential for efficient adenoviral replication in cells containing functional p53. Hence, adenovirus variants which substantially lack the ability to bind p53 are replication deficient in non-replicating, non-neoplastic cells having normal levels of functional p53.

Human tumor cells frequently are homozygous or heterozygous for mutated (e.g., substitution, deletion, frameshift mutants) p53 alleles, and lack p53 function necessary for normal control of the cell cycle (Hollstein et al. (1991) *Science* 253: 49; Levine et al. (1991) op. cit., incorporated herein by reference). Thus, many neoplastic cells are p53^{-/-}, either because they lack sufficient levels of p53 protein and/or because they express mutant forms of p53 which are

incapable of substantial p53 function, and which may substantially diminish p53 function even when wild-type p53 may be present (e.g., by inhibiting formation of functional multimers). Some neoplastic cells may comprise alleles encoding essentially wild-type p53 proteins, but may comprise a second site mutation that substantially abrogates p53 function, such as a mutation that results in p53 protein being localized in the cytoplasm rather than in the nucleus; such second site mutants also substantially lack p53 function.

It is believed that replication deficient adenovirus species which lack the capacity to complex p53 but substantially retain other essential viral replicative functions will exhibit a replication phenotype in cells which are deficient in p53 function (e.g., cells which are homozygous for substantially deleted p53 alleles, cells which comprise mutant p53 proteins which are essentially nonfunctional) but will not substantially exhibit a replicative phenotype in non-replicating, non-neoplastic cells. Such replication deficient adenovirus species are referred to herein for convenience as E1b-p53^{-/-} replication deficient adenoviruses.

A cell population (such as a mixed cell culture or a human cancer patient) which comprises a subpopulation of neoplastic cells lacking p53 function and a subpopulation of non-neoplastic cells which express essentially normal p53 function can be contacted under infective conditions (i.e., conditions suitable for adenoviral infection of the cell population, typically physiological conditions) with a composition comprising an infectious dosage of a E1b-p53^{-/-} replication deficient adenovirus. Such contacting results in infection of the cell population with the E1b-p53^{-/-} replication deficient adenovirus. The infection produces preferential expression of a replication phenotype in a significant fraction of the cells comprising the subpopulation of neoplastic cells lacking p53 function but does not produce a substantial expression of a replicative phenotype in the subpopulation of non-neoplastic cells having essentially normal p53 function. The expression of a replication phenotype in an infected p53^{-/-} cell results in the death of the cell, such as by cytopathic effect (CPE), cell lysis, apoptosis, and the like, resulting in a selective ablation of neoplastic p53^{-/-} cells from the cell population.

Typically, E1b-p53^{-/-} replication deficient adenovirus constructs suitable for selective killing of p53^{-/-} neoplastic cells comprise mutations (e.g., deletions, substitutions, frameshifts), which inactivate the ability of the E1b p55 polypeptide to bind p53 protein effectively. Such inactivating mutations typically occur in the regions of p55 which bind p53. Optionally, the mutant E1b region may encode and express a functional p19 protein encoded by the E1b region remains and that is functional in transactivation of adenoviral early genes in the absence of E1a polypeptides.

Suitable E1b-p53^{-/-} replication deficient adenovirus constructs for use in the methods and compositions of the invention include, but are not limited to the following examples: (1) adenovirus type 2 dl 1520, which contains a C to T mutation at nucleotide position 2022 that generates a stop codon 3 amino acids downstream of the AUG codon used for initiation of translation of the p55 protein and a deletion between nucleotides 2496 and 3323 replaced with a small linker insertion that generates a second stop codon at nucleotide 3336; the expression of the p19 protein is essentially unaffected (Barker and Berk (1987) *Virology* 156: 107, incorporated herein by reference, and (2) a composite adenovirus construct comprising adenovirus type 2 dl 1520 comprising at least the position 2022 mutation and/or the 2496-3323 deletion mutation, or a substantial portion thereof, and an additional mutation in p19 to yield a p19 cyt

mutant; the composite virus construct lacks p55 and comprises the enhanced cytopathic effect of the p19 cyt mutation. Ad2 dl 1520 are available from Dr. A. Berk, University of California at Los Angeles, Los Angeles, Calif., and are described in the literature, including Barker and Berk (1987) *Virology* 156: 107, incorporated herein by reference.

It may be preferable to incorporate additional mutations into such adenovirus constructs to inhibit formation of infectious virions in neoplastic cells which otherwise would support replication of the E1b-p53^{-/-} mutants. Such additional inactivating mutations would be preferred in therapeutic modalities wherein complete viral replication forming infectious virions capable of spreading to and infecting adjacent cells is undesirable. These fully inactivated mutants are referred to as nonreplicable, E1b-P53^{-/-} mutants. Such nonreplicable mutants comprise mutations which prevent formation of infectious virions even in p53^{-/-}RB^{-/-} cells; such mutations typically are structural mutations in an essential virion protein or protease.

However, in many modalities it is desirable for the mutant virus to be replicable and to form infectious virions containing the mutant viral genome which may spread and infect other cells, thus amplifying the antineoplastic action of an initial dosage of mutant virus.

Additional E1b^{-/-} mutants lacking the capacity to bind p53 can be generated by those of skill in the art by generating mutations in the E1b gene region encoding the p55 polypeptide, expressing mutant p55 polypeptides, contacting the mutant p55 polypeptides with p53 or a binding fragment of p53 under aqueous binding conditions, and identifying mutant E1b polypeptides which do not specifically bind p53 as being candidate E1b^{-/-} mutants suitable for use in the invention.

More typically, a functional assay will be used to identify candidate E1b^{-/-} mutants. For example, the Friend assay for determination of p53 function will be performed essentially as described in Prebourg et al. (1992) *Cancer Res.* 52: 6977, incorporated herein by reference. E1b mutants which lack the capacity to inactivate p53 will be identified as candidate E1b^{-/-} replication deficient mutants.

E1a/E1b Double Mutants

Some human tumor cells lack both p53 function and RB function, either by mutational inactivation or deletion of one or both protein species. Such cells are termed p53^{-/-}RB^{-/-} cells.

It is believed that replication deficient adenovirus species which lack the capacity to bind p53 and which also lack the capacity to bind RB, but which substantially retain other essential viral replicative functions will preferentially exhibit a replication phenotype in p53^{-/-}RB^{-/-} cells. Such replication deficient adenovirus species are referred to herein for convenience as E1a-RB^{-/-}/E1b-P53^{-/-} replication deficient adenoviruses, or simply E1a/E1b double mutants. Such E1a/E1b double mutants can be constructed by those of skill in the art by combining at least one E1a-RB^{-/-} mutation in the E1a region and at least one E1b-p53^{-/-} mutation in E1b region encoding p55 to form an E1a/E1b double mutant adenovirus. Such a replication deficient double mutant adenovirus will exhibit a replication phenotype in cells which are deficient in both p53 and RB functions but will not substantially exhibit a replicative phenotype in nonreplicating, non-transformed cells or in cells which are deficient in either p53 or RB function but not both functions. For example, the Ad5 dl 434 mutant (Grodzicker et al. (1980) *Cell* 21: 454, incorporated herein

by reference) comprises a deletion of the E1a locus and a partial deletion of the E1b locus, and substantially lacks the capacity to encode functional E1a and E1b p55 proteins.

A cell population (such as a mixed cell culture or a human cancer patient) which comprises a subpopulation of neoplastic cells lacking p53 and RB functions and a subpopulation of non-neoplastic cells which express essentially normal p53 function and/or RB function can be contacted under infective conditions (i.e., conditions suitable for adenoviral infection of the cell population, typically physiological conditions) with a composition comprising an infectious dosage of a replication deficient E1a/E1b double mutant adenovirus. Such contacting results in infection of the cell population with the E1a/E1b double mutant replication deficient adenovirus. The infection produces preferential expression of a replication phenotype in a significant fraction of the cells comprising the subpopulation of neoplastic cells lacking both p53 function and RB function but does not produce a substantial expression of a replicative phenotype in the subpopulation of non-neoplastic cells having essentially normal p53 function and/or RB function. The expression of a replication phenotype in an infected p53^{-/-}RB^{-/-} cell results in the death of the cell, such as by cytopathic effect (CPE), cell lysis, and the like, resulting in a selective ablation of neoplastic p53^{-/-}RB^{-/-} cells from the cell population.

It may be preferable to incorporate additional mutations into such adenovirus constructs to inhibit formation of infectious virions in neoplastic cells which otherwise would support replication of an E1a/E1b double mutant. Such additional inactivating mutations would be preferred in therapeutic modalities wherein complete viral replication forming infectious virions capable of spreading to and infecting adjacent cells is undesirable. These fully inactivated mutants are referred to as nonreplicable E1a/E1b double mutants. Such nonreplicable mutants comprise mutations which prevent formation of infectious virions even in p53^{-/-}RB^{-/-} cells; such mutations typically are structural mutations in an essential virion protein or protease.

However, in many modalities it is desirable for the mutant virus to be replicable and to form infectious virions containing the mutant viral genome which may spread and infect other cells, thus amplifying the antineoplastic action of an initial dosage of mutant virus.

Negative Selection Viral Constructs

Although expression of an adenoviral replication phenotype in an infected cell correlates with viral-induced cytotoxicity, generally by cell lysis, cytopathic effect (CPE), apoptosis, or other mechanisms of cell death, it may often be preferable to augment the cytotoxicity of a recombinant adenovirus that is to be used for antineoplastic therapy. Such augmentation may take the form of including a negative selection gene in the recombinant adenovirus, typically operably linked to an adenoviral promoter which exhibits positive transcriptional modulation in cells expressing a replication phenotype. For example, a HSV tk gene cassette may be operably linked immediately downstream of an E3 promoter of a replication deficient adenovirus, such as Ad5 NT dl 1110. Frequently, it is desirable to delete a nonessential portion (i.e., for viral replication and packaging) of the adenoviral genome to accommodate the negative selection cassette; thus a substantial portion of the E3 gene region may be deleted and replaced with a negative selection cassette such as an HSV tk gene operably linked to an E2 promoter (and enhancer) or other suitable promoter/enhancer.

Alternatively, a negative selection gene may be operably linked to an adenovirus late region promoter to afford efficient expression of the negative selection gene product in cells expressing a replication phenotype characterized by transcription from late gene promoters.

For embodiments where viral replication forming infectious virions in vivo is undesirable, adenovirus replication deficient constructs which are nonreplicable are used. Such nonreplicable mutants comprise an E1a⁽⁻⁾ and/or E1b⁽⁻⁾ mutation and comprise all genetic functions necessary for generating a replication phenotype in a suitable neoplastic cell (e.g., a p53⁽⁻⁾ cell, a RB⁽⁻⁾ cell, or a p53⁽⁻⁾RB⁽⁻⁾ cell) but have deleted at least one essential gene function necessary for formation of infectious virions, such as structural coat proteins, proteases, and the like. Alternatively, an elicited immune response evoked by the virus may neutralize infectious virions and moderate spread of the viral infection. Nonreplicable mutants lacking a complementable trans-acting function in addition to an E1a and/or E1b mutation may be propagated in conjunction with a complementary helper virus or a helper cell line capable of providing the deleted trans-acting function(s). For example, the 293 cell line (ATCC # CRL 1573; Graham et al. (1977) *J. Gen. Virol.* 36: 59, incorporated herein by reference) which provides E1a and E1b functions in trans may be modified to provide additional functions in trans, such as a viral coat protein or the like, to permit propagation of the "nonreplicable" mutants for developing virus stocks.

Expression of the HSV tk gene in a cell is not directly toxic to the cell, unless the cell is exposed to a negative selection agent such as gancyclovir or FIAU. Infected cells expressing a replication phenotype wherein a negative selection gene is substantially expressed may produce essentially no additional cytotoxicity until the negative selection agent (e.g., gancyclovir) is administered in an effective selective dosage, at which time the infected cells expressing the tk gene will be selectively ablated; thus negative selection can be used for enhanced cytopathic killing and/or to damp out further viral replication by killing cells exhibiting a replicative phenotype. Further, by adjusting the dosages and/or administration schedule of the negative selection agent, it is possible to produce only a partial ablation of the infected cell population expressing the negative selection gene. Generally, the dosage of gancyclovir is calibrated by generating a standard dose-response curve and determining the dosage level at which a desired level of ablation of infected neoplastic cells is observed. Information regarding administration of gancyclovir (GANC) to animals is available in various sources in the art, including human prescribing directions from package inserts. When used in cell culture, a selective concentration of gancyclovir is typically in the range of 0.05 μ M to 50 μ M, typically about 1 μ M, with about 0.2 μ M used for in vitro applications and about 1-5 μ M administered for in vivo applications (typically administered over about 24 hours by continuous infusion from an osmotic pump loaded with 125 mg/ml of gancyclovir in aqueous solution). A dosage schedule for in vivo administration may comprise gancyclovir at a dosage of 5 mg/kg bodyweight B.I.D., given intravenously for seven days.

Negative selection genes may be incorporated into E1a-RB⁽⁻⁾ replication deficient adenovirus constructs, E1a-p53⁽⁻⁾ replication deficient adenoviral constructs, E1a/E1b double mutant replication deficient viral constructs, or the like. A preferred embodiment is an HSV tk gene cassette (Zijlstra et al. (1989) *Nature* 342:435; Mansour et al. (1988) *Nature* 336: 348; Johnson et al. (1989) *Science* 245: 1234; Adair et al. (1989) *Proc. Natl. Acad. Sci. (U.S.A.)* 86: 4574;

Capecchi, M. (1989) *Science* 244:1288, incorporated herein by reference) operably linked to the E2 promoter of A ϕ 5 NT dl1110 or an alternative promoter and/or enhancer (e.g., major late promoter, E1a promoter/enhancer, E1b promoter/enhancer), with a polydenylation site to form a tk expression cassette. The tk expression cassette (or other negative selection expression cassette) is inserted into the adenoviral genome, for example, as a replacement for a substantial deletion of the E3 gene. Other negative selection genes and replication deficient adenovirus constructs will be apparent to those of skill in the art. It is believed that a negative selection gene operably linked to the E2 promoter is an especially preferred embodiment for incorporation into E1a⁽⁻⁾ replication-deficient adenovirus mutants, as the E2 promoter contains multiple E2F sites, whereas RB⁽⁻⁾ and p53⁽⁻⁾RB⁽⁻⁾ lack RB function and presumably will exhibit more efficient transcription from the E2 promoter.

Diagnostic and In Vitro Uses

The replication deficient adenoviruses of the invention may be used to detect the presence of cells lacking p53 and/or RB function. For example, a cell sample comprising a subpopulation of neoplastic cells lacking p53 and/or RB can be infected with a suitable replication deficient adenovirus species. After a suitable incubation period, the cells in the cell sample that express a replication phenotype (e.g., loss of ability to exclude Trypan blue, virion formation, ³H-thymidine incorporation into viral DNA) can be quantified to provide a measure of the number or proportion of replicative and/or neoplastic cells in the cell sample. Such methods may be used to diagnose neoplasms and/or evaluate tumor cell load following chemotherapy in a patient on the basis of an explanted cell sample (e.g., a lymphocyte sample from a patient undergoing chemotherapy for a lymphocytic leukemia).

Alternative diagnostic uses and variations are apparent; for example, a reporter gene (e.g., luciferase, β -galactosidase) may be substituted for a negative selection gene in a replication deficient adenovirus; transformed cells may be scored (such as in a cellular sample or transformation assay) by the expression of the reporter gene, which is correlated with expression of a replication phenotype indicating a lack of p53 and/or RB in a cell.

Therapeutic Methods

Therapy of neoplastic disease may be afforded by administering to a patient a composition comprising replication defective adenoviruses of the invention, including: E1a-RB⁽⁻⁾ replication deficient adenoviruses, E1b p53⁽⁻⁾ replication deficient adenoviruses, E1a/E1b double mutants, and replication deficient adenoviruses further comprising a negative selection gene.

Various human neoplasms comprising cells that lack p53 and/or RB functions may be treated with the replication deficient adenoviral constructs. For example but not limitation, a human patient or nonhuman mammal having a bronchogenic carcinoma, nasopharyngeal carcinoma, laryngeal carcinoma, small cell and non-small cell lung carcinoma, lung adenocarcinoma, hepatocarcinoma, pancreatic carcinoma, bladder carcinoma, colon carcinoma, breast carcinoma, cervical carcinoma, ovarian carcinoma, or lymphocytic leukemias may be treated by administering an effective antineoplastic dosage of an appropriate replication deficient adenovirus. Suspensions of infectious adenovirus particles may be applied to neoplastic tissue by various routes, including intravenous, intraperitoneal, intramuscular,

subdermal, and topical. A adenovirus suspension containing about 10^3 to 10^{12} or more virions per ml may be inhaled as a mist (e.g., for pulmonary delivery to treat bronchogenic carcinoma, small-cell lung carcinoma, non-small cell lung carcinoma, lung adenocarcinoma, or laryngeal cancer) or swabbed directly on a tumor site for treating a tumor (e.g., bronchogenic carcinoma, nasopharyngeal carcinoma, laryngeal carcinoma, cervical carcinoma) or may be administered by infusion (e.g., into the peritoneal cavity for treating ovarian cancer, into the portal vein for treating hepatocarcinoma or liver metastases from other non-hepatic primary tumors) or other suitable route, including direct injection into a tumor mass (e.g., a breast tumor), enema (e.g., colon cancer), or catheter (e.g., bladder cancer).

Candidate antineoplastic adenovirus mutants may be further evaluated by their capacity to reduce tumorigenesis or neoplastic cell burden in nu/nu mice harboring a transplant of neoplastic cells lacking p53 and/or RB function, as compared to untreated mice harboring an equivalent transplant of the neoplastic cells.

Antineoplastic replication deficient adenovirus mutants may be formulated for therapeutic and diagnostic administration to a patient having a neoplastic disease. For therapeutic or prophylactic uses, a sterile composition containing a pharmacologically effective dosage of one or more species of antineoplastic replication deficient adenovirus mutant is administered to a human patient or veterinary nonhuman patient for treatment of a neoplastic condition. Generally, the composition will comprise about 10^3 to 10^{15} or more adenovirus particles in an aqueous suspension. A pharmaceutically acceptable carrier or excipient is often employed in such sterile compositions. A variety of aqueous solutions can be used, e.g., water, buffered water, 0.4% saline, 0.3% glycine and the like. These solutions are sterile and generally free of particulate matter other than the desired adenoviral virions. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate, etc. Excipients which enhance infection of cells by adenovirus may be included.

Replication deficient viruses may be delivered to neoplastic cells by liposome or immunoliposome delivery; such delivery may be selectively targeted to neoplastic cells on the basis of a cell surface property present on the neoplastic cell population (e.g., the presence of a cell surface protein which binds an immunoglobulin in an immunoliposome). Typically, an aqueous suspension containing the virions are encapsulated in liposomes or immunoliposomes. For example, a suspension of replication deficient adenovirus virions can be encapsulated in micelles to form immunoliposomes by conventional methods (U.S. Pat. No. 5,043,164, U.S. Pat. No. 4,957,735, U.S. Pat. No. 4,925,661; Connor and Huang (1985) *J. Cell Biol.* 101: 582; Lasic DD (1992) *Nature* 355: 279; *Novel Drug Delivery* (eds. Prescott, L. F. and Nimmo, W. S.: Wiley, New York, 1989); Reddy et al. (1992) *J. Immunol.* 148: 1585; incorporated herein by reference). Immunoliposomes comprising an antibody that binds specifically to a cancer cell antigen (e.g., CALLA, CEA) present on the cancer cells of the individual may be used to target virions to those cells.

The compositions containing the present antineoplastic replication deficient adenoviruses or cocktails thereof can be administered for prophylactic and/or therapeutic treatments of neoplastic disease. In therapeutic application, compositions are administered to a patient already affected by the

particular neoplastic disease, in an amount sufficient to cure or at least partially arrest the condition and its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose" or "efficacious dose." Amounts effective for this use will depend upon the severity of the condition, the general state of the patient, and the route of administration.

In prophylactic applications, compositions containing the antineoplastic replication deficient adenoviruses or cocktails thereof are administered to a patient not presently in a neoplastic disease state to enhance the patient's resistance to recurrence of a neoplasm or to prolong remission time. Such an amount is defined to be a "prophylactically effective dose." In this use, the precise amounts again depend upon the patient's state of health and general level of immunity.

Single or multiple administrations of the compositions can be carried out with dose levels and pattern being selected by the treating physician. In any event, the pharmaceutical formulations should provide a quantity of the antineoplastic replication deficient adenoviruses of this invention sufficient to effectively treat the patient.

Antineoplastic replication deficient adenoviral therapy of the present invention may be combined with other antineoplastic protocols, such as conventional chemotherapy.

Propagation of Mutant Adenovirus

Adenoviral mutants of the invention (e.g., E1a-RB⁽⁻⁾ replication deficient adenoviruses, E1b-p53⁽⁻⁾ replication deficient adenoviruses, and E1a/E1b double mutants) typically are propagated as viral stocks in a cell line (e.g., the 293 cell line ATCC # CRL 1573, American Type Culture Collection, Rockville, Md.; Graham et al. (1977) *J. Gen. Virol.* 36: 59) which can provide E1a function, E1b function, or both E1a and E1b functions, respectively, in trans to support replication and formation of infectious mutant virions.

The following examples are offered by way of example and not by way of limitation. Variations and alternate embodiments will be apparent to those of skill in the art.

EXPERIMENTAL EXAMPLE

The following experimental example demonstrates that administering a replication-deficient recombinant adenovirus preparation to neoplastic cells lacking p53 and/or Rb function can effectively kill neoplastic cells. The example also shows that non-neoplastic cells containing p53 and Rb function are relatively resistant to killing by the replication-deficient recombinant adenovirus preparation. Therefore, the data presented hereinbelow provide experimental verification that administration of replication-deficient recombinant adenovirus can be used to selectively kill neoplastic cells. The selective killing is provided by exploiting the differential ability of the mutant adenoviruses to induce a replication phenotype in neoplastic cells, but substantially not induce a replication phenotype (or associated cytopathic effect) in non-neoplastic cells.

Control non-neoplastic cells and cell lines representing a variety of neoplastic cell types were plated in 6-well culture dishes at or near confluence in DMEM (high glucose) with 10% fetal bovine serum and incubated at 37° C., 5% CO₂ under standard culturing conditions. Cells were plated to be screened at a density of 5×10^5 cells/well. The neoplastic cell lines tested were: SAOS-2 (ATCC HTB85), derived from a human primary osteogenic sarcoma; U-2OS (ATCC HTB96), derived from a human osteogenic sarcoma;

HS700T, (ATCC HTB147), derived from a human metastatic adenocarcinoma originating in the pancreas or intestines; 293 (ATCC CRL1573), transformed human embryonal kidney; and DLD-1 (ATCC CCL221), derived from a human colon adenocarcinoma. Each of the cell lines is available from American Type Culture Collection, Rockville, Md. The control non-neoplastic cells were IMR90 (ATCC CCL 186) and WI-38 (ATCC CCL 75), both diploid human lung fibroblast lines.

These cell cultures were subsequently infected, in parallel, with wild-type adenovirus Type 2, and replication-deficient recombinant adenovirus mutants dl 11010, dl 434, and dl 1520. An extra culture dish was plated for the purpose of counting cells. These cells came from the same suspension of cells used for the viral infections. Cells were counted in order to determine the number of viral plaque-forming units (PFU) to add to the cell cultures for a desired multiplicity of infection (MOI). The wild-type adenovirus Type 2 and the mutant adenoviruses were added to the parallel cell cultures at MOIs of 0.1, 1.0, 10, and 100. Virus suspended in PBS was added to the cell wells in a volume of 1 ml. The inoculated culture dishes were rocked in both the X- and Y-axes immediately after inoculation and halfway through the adsorption time of approximately one hour at 37° C., 5% CO₂. After the one hour adsorption period, 2 ml of DMEM with high glucose and 2% fetal bovine serum was added, and the cultures incubated at 37° C., 5% CO₂ under standard culturing conditions.

At various times after infection, see, FIGS. 2(a-i), cell cultures were stained with 0.5% crystal violet in 20% methanol in order to determine the efficacy of cell killing by the virus preparations. Dead cells were detached and rinsed out of the wells, whereas living cells remained in the well and were stained with the dye. The results demonstrate that the replication-deficient recombinant adenovirus preparations were able to preferentially kill neoplastic cells as compared to non-neoplastic cells, and that wild-type adenovirus Type 2 killed both neoplastic and non-neoplastic cells approximately equally well. The results demonstrate that the dl 11010 mutant was particularly effective at killing neoplastic cells, with the dl 1520 mutant and the dl 434 mutant also being effective. An example of the results is shown in FIGS. 2a-i, which are photographs of the stained cell wells obtained at various times after infection with wild-type adenovirus 2, the mutants: dl 1010 (E1A⁻), dl1520 (E1B⁻), or dl 434. FIG. 2a shows that in the absence of virus, each of the cell cultures had grown to confluence and were attached to the culture wells. FIGS. 2b-c shows that wild-type adenovirus 2 was able to kill cells in each of the culture wells, although with variable efficacy and incompletely, with at least some cells staining in each well. Note that WI-38 and SAOS-2 lines are not good hosts for adenovirus infection and that IMR90 (FIGS. 3a-c) serves as an alternate control cell line for human diploid fibroblasts. FIGS. 2d-e shows that the dl 1010 mutant was able to kill effectively all of the neoplastic cell lines except the infection-resistant SAOS-2. dl 1010 killed the 293, U2OS, HS700T, and DLD-1 lines by 12 days after infection and did not substantially kill diploid human lung fibroblasts (WI-38). FIGS. 2f-g shows that the dl 1520 mutant was able to kill effectively 3 of the 5 neoplastic cell lines (293, HS700T, and DLD-1) by 14-20 days after infection and did not substantially kill diploid human lung fibroblasts (WI-38). dl 1520 is an E1B⁻ mutant, and cell line U2OS does not allow such an E1B⁻ mutant virus to replicate, indicating specificity of the different transformed cell lines for infection by the mutant recombination-defective adenoviruses, as predicted. FIGS.

2h-i shows that the dl 434 mutant (a double-mutant: E1A⁻E1B⁻) was able to kill effectively 3 of the 5 neoplastic cell lines (293, HS700T, and DLD-1) by 14-20 days after infection and did not substantially kill diploid human lung fibroblasts (WI-38). The DLD-1 and U2OS lines displayed a partially resistant phenotype for replication of dl 434.

FIGS. 3(a-c) show that IMR90 human diploid lung fibroblasts were killed differentially by wild-type Ad2, dl 1010, and dl 1520. The 293 cell line serves as a positive control. FIG. 3(a) shows that wild-type Ad2 killed the IMR90 cells effectively at an MOI of 10 or 100. FIG. 3(b) shows that dl 1010 was unable to replicate in IMR90 cells at the highest MOI tested, even though dl 1010 killed neoplastic cell lines 293, U2OS, HS700T, and DLD-1 as shown in FIGS. 2(d-e). FIG. 3(c) shows that dl 1520 virus was able to kill IMR90 cells effectively only at an MOI of 100; at this high dosage of virus the possibility that the cells die as a result of virus overload rather than replication cannot be ruled out.

Thus, the data shown in FIGS. 2(a-i) and 3(a-c) indicate that replication-deficient recombinant adenovirus mutants can be used to selectively kill neoplastic cells lacking p53 and/or Rb function, as predicted.

Although the present invention has been described in some detail by way of illustration for purposes of clarity of understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the claims.

I claim:

1. A method for ablating neoplastic cells in a cell population, comprising the steps of:

contacting under infective conditions (1) a recombinant replication deficient adenovirus lacking an expressed viral oncoprotein capable of binding a functional p53 tumor suppressor gene product, with (2) a cell population comprising non-neoplastic cells containing said functional p53 tumor suppressor gene product which forms a bound complex with a viral oncoprotein and neoplastic cells lacking said functional p53 tumor suppressor gene product, thereby generating an infected cell population.

2. A method according to claim 1, wherein the viral oncoprotein is an adenovirus E1b polypeptide.

3. A method according to claim 1, wherein the neoplastic cells are p53⁻RB⁻.

4. A method according to claim 3, wherein the recombinant replication deficient adenovirus does not encode an E1a polypeptide capable of binding RB and also does not encode an E1b p55 polypeptide capable of binding p53.

5. A method according to claim 1, wherein the recombinant replication deficient adenovirus is selected from the group consisting of: Ad5 dl434 and Ad2 dl1520.

6. A method according to claim 5, wherein the recombinant replication deficient adenovirus is Ad5 dl434.

7. A method according to claim 5, wherein the recombinant replication deficient adenovirus is Ad2 dl1520.

8. A method according to claim 1, wherein said cell population comprising neoplastic cells and non-neoplastic cells is present in a mammal and said contacting step is performed in vivo by administering the recombinant replication deficient adenovirus to a mammal.

9. A method according to claim 8, wherein the mammal is a human.

10. A method according to claim 1, wherein the recombinant replication deficient adenovirus can be replicated to form infectious virions in a neoplastic cell lacking p53 function, or lacking both p53 function and RB function.

11. A method according to claim 10, wherein the infectious virions formed in the neoplastic cell are able to spread and infect adjacent cells in vivo in a patient.

12. A method according to claim 1, wherein the recombinant replication deficient adenovirus is nonreplicable for forming infectious virions in non-neoplastic cells of a human patient.

13. A method according to claim 1, wherein the recombinant replication deficient adenovirus is an E1a/E1b double mutant.

14. A method according to claim 1, wherein the recombinant replication deficient adenovirus is an E1b mutant.

15. A method for treating a neoplastic condition in a human, comprising administering a composition comprising a therapeutically effective dosage of recombinant replication deficient adenovirus to a human patient having a neoplasm comprising neoplastic cells lacking a functional p53 tumor suppressor gene product.

16. A method according to claim 15, wherein the tumor suppressor gene product is p53 and the composition comprises a recombinant replication deficient adenovirus that is a E1b-p53⁽⁻⁾ replication deficient adenovirus.

17. A method according to claim 16, wherein the composition comprises a recombinant replication deficient adenovirus that is an E1a/E1b double mutant replication deficient adenovirus.

18. A method according to claim 15, wherein the composition comprises a recombinant replication deficient adenovirus selected from the group consisting of E1b-p53⁽⁻⁾ replication deficient adenovirus and E1a/E1b double mutant replication deficient adenovirus, and further comprising a negative selection gene operably linked to a viral promoter.

19. A method according to claim 18, wherein the virus is selected from the group consisting of Ad5 dl434 and Ad2 dl1520.

20. A method according to claim 19, wherein the recombinant replication deficient adenovirus is Ad5 dl434.

21. A method according to claim 19, wherein the recombinant replication deficient adenovirus is Ad2 dl1520.

22. A method for treating a neoplastic condition with a replication deficient virus comprising administering to a patient with a neoplasm an effective dose of a recombinant replication deficient adenovirus or papillomavirus whose replication defective phenotype depends on the presence of a p53 tumor suppressor gene product.

23. A method according to claim 22, wherein the virus is a human papillomavirus comprising a mutation in an E6 gene.

24. A method according to claim 22, wherein the virus is adenovirus.

25. A method according to claim 24, wherein the adenovirus lacks an E1b polypeptide capable of binding a functional p53 tumor suppressor gene product.

26. A method according to claim 24, wherein the recombinant replication deficient adenovirus is selected from the group consisting of: Ad5 dl434 and Ad2 dl1520.

27. A method according to claim 26, wherein the recombinant replication deficient adenovirus is Ad5 dl434.

28. A method according to claim 26, wherein the recombinant replication deficient adenovirus is Ad2 dl1520.

29. A method for ablating neoplastic cells in a cell population, comprising the steps of:

contacting under infective conditions (1) a recombinant replication deficient adenovirus or papillomavirus whose replication defective phenotype depends on the presence of a functional p53 host tumor suppressor gene product, with (2) a cell population comprising non-neoplastic cells containing said functional tumor suppressor gene product which forms a bound complex with a viral oncoprotein and neoplastic cells lacking said functional tumor suppressor gene product, thereby generating an infected cell population.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 5,677,178
DATED : October 14, 1997
INVENTOR(S) : Francis McCormick

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 22, claim 29, line 27, substitute "repletion" with --replication--.

Signed and Sealed this
Twenty-fourth Day of February, 1998

Attest:



BRUCE LEHMAN

Attesting Officer

Commissioner of Patents and Trademarks

Phase II Trial of Intratumoral Administration of ONYX-015, a Replication-Selective Adenovirus, in Patients With Refractory Head and Neck Cancer

By J. Nemunaitis, F. Khuri, I. Ganly, J. Arseneau, M. Posner, E. Vokes, J. Kuhn, T. McCarty, S. Landers, A. Blackburn, L. Romel, B. Randle, S. Kaye, and D. Kim

Purpose: To determine the safety, humoral immune response replication, and activity of multiple intratumoral injections of ONYX-015 (replication selective adenovirus) in patients with recurrent squamous cell carcinoma of the head and neck (SCCHN).

Patients and Methods: This phase II trial enrolled patients with SCCHN who had recurrence/relapse after prior conventional treatment. Patients received ONYX-015 at a dose of 2×10^{11} particles via intratumoral injection for either 5 consecutive days (standard) or twice daily for 2 consecutive weeks (hyperfractionated) during a 21-day cycle. Patients were monitored for tumor response, toxicity, and antibody formation.

Results: Forty patients (30 standard and 10 hyperfractionated) received 533 injections of ONYX-015. Standard treatment resulted in 14% partial to complete regression, 41% stable disease, and 45% progressive disease rates. Hyperfractionated treatment resulted in

10% complete response, 62% stable disease, and 29% progressive disease rates. Treatment-related toxicity included mild to moderate fever (67% overall) and injection site pain (47% on the standard regimen, 80% on the hyperfractionated regimen). Detectable circulating ONYX-015 genome suggestive of intratumoral replication was identified in 41% of tested patients on days 5 and 6 of cycle 1; 9% of patients had evidence of viral replication 10 days after injection during cycle 1, and no patients had evidence of replication ≥ 22 days after injection.

Conclusion: ONYX-015 can be safely administered via intratumoral injection to patients with recurrent/refractory SCCHN. ONYX-015 viremia is transient. Evidence of modest antitumoral activity is suggested.

J Clin Oncol 19:289-298. © 2001 by American Society of Clinical Oncology.

THE PROGNOSIS FOR recurrent squamous cell carcinoma of the head and neck (SCCHN) region is discouraging.^{1,2} Local tumor progression leads to morbidity and even death in the majority of patients. Therefore, improved local and local-regional therapeutic approaches are needed. Treatment after failure of surgery and radiation therapy generally involves chemotherapy.^{1,2} Approximately 30% to 40% of patients with recurrent head and neck cancer respond to combination chemotherapy, which generally includes cisplatin. The duration of response is short, and median survival is less than 6 months.¹⁻⁹ Furthermore, local expansion of disease during or after chemotherapy leads to devastating functional, economic, cosmetic, and psychologic effects to the patient. Because recurrence frequently occurs within a prior radiation field, further radiotherapy is not an option, and palliative surgery is generally associated with excess morbidity and additional cost, while not affecting survival. Second-line chemotherapy with other agents, such as paclitaxel, docetaxel, methotrexate, topotecan, or gemcitabine (alone or in combination), has been tested, but response rates remain poor,¹⁰⁻³¹ and the duration of the response is less than a few months.^{9,32,33} Therefore, novel approaches to the local control of chemotherapy resistant/refractory SCCHN are needed.

ONYX-015 (dl1520) is a replication-selective adenovirus.³⁴ Efficient adenovirus replication is dependent on the

expression of proteins that inactivate *p53*.^{35,36} The normal *p53* gene product inhibits viral replication. ONYX-015 is an adenovirus that has been modified by deletion of the E1B 55-kd DNA fragment. The E1B-55-kd gene product inactivates *p53* in complex with E4ORF6.³⁷ It has been hypothesized that deletion of the E1B-55-kd region enables the *p53* protein to maintain its function, thereby inhibiting viral replication in cells with normal *p53* function; however, in cells that lack normal *p53* function, such as malignant cells, the E1B-55-kd gene product may be expendable and the cells should be susceptible to replication and killing after infection.

Initial reports³⁸ suggested that *p53* mutant tumor cells could be lysed in a replication-dependent fashion both in

From US Oncology, Dallas, Baylor University Medical Center, Dallas, and M.D. Anderson Cancer Center, Houston, TX, Beatson Oncology Centre, Glasgow, Scotland, Albany Regional Cancer Center, Albany, NY, Dana-Farber Cancer Institute, Boston, MA, University of Chicago, Chicago, IL, and Onyx Pharmaceuticals, Inc, Richmond, CA.

Submitted June 5, 2000; accepted November 9, 2000.

Address reprint requests to John Nemunaitis, MD, US Oncology, 3535 Worth St, Collins Bldg, 5th Floor, Dallas, TX 75246; email John.Nemunaitis@USOncology.com.

© 2001 by American Society of Clinical Oncology.
0732-183X/01/1902-289

vitro and in vivo after exposure to ONYX-015.^{34,39} In addition, several tumor lines containing a normal wild-type *p53* gene sequence were also found to be sensitive to the oncolytic activity of ONYX-015.³⁹⁻⁴¹ This finding is expected, since *p53* function can be lost through multiple mechanisms besides gene mutation (eg, *p53* protein binding degradation). Importantly, most groups found significantly less replicative capacity of ONYX-015 in weak normal cells compared with malignant cells,^{39,41,42} which suggests a possible therapeutic index to ONYX-015 in the treatment of cancer.

Phase I investigation identified the toxicity of intratumoral injection of ONYX-015 to be limited to transient low-grade fever and injection site pain in one third of patients (S. Kaye, manuscript in preparation). Viral doses up to 1×10^{11} plaque-forming units (pfu) given daily once every 3 weeks, or 1×10^{10} pfu for 5 consecutive days every 3 weeks, were well-tolerated. No dose-limiting toxicity or maximum-tolerated dose was identified. Dose escalation, therefore, proceeded to the highest dose that could be practically manufactured. Additionally, multiday dosing with each dose administered to separate tumor quadrants seemed to be associated with a more effective induction of tumor necrosis over single-day dosing. Thus, we initiated a phase II investigation with ONYX-015 to be administered by intratumoral injection with multiple doses per cycle to patients with recurrent or refractory SCCHN.

PATIENTS AND METHODS

Enrollment Criteria

Patients were required to have histologically confirmed SCCHN (excluding nasopharyngeal) that had (1) recurred/relapsed after surgery and/or radiotherapy for the primary tumor and (2) had progressed on or within 8 weeks after completion of chemotherapy and/or radiotherapy (ie, tumors were refractory). Tumors could not be surgically curable. The tumor mass to be treated with ONYX-015 had to be adequately injectable (as defined below) and measurable (radiographically or by physical examination). Patients had to be older than 18 years old and had to have a Karnofsky performance status score of ≥ 70 and life expectancy of ≥ 3 months. Normal hematologic function and renal function were also required. A signed consent form (internal review board-approved) was required before enrollment. The *p53* gene status was not used as an enrollment criterion. Institutional review board approval of the protocol and consent form was required.

Baseline Assessment

Baseline assessments were made before treatment. Baseline *p53* gene sequencing and immunohistochemistry were performed on paraffin-embedded or frozen (-70°C) tumor material used for diagnosis of recurrence (when available). Baseline blood tests were performed that included complete blood counts, CD3, CD4, and CD8 lymphocyte counts, electrolytes, blood urea nitrogen, creatinine, and liver function tests. In addition, baseline neutralizing antibody titers to ONYX-015 were determined (most adults have neutralizing antibodies to the adenovirus type 5 coat proteins that are present on ONYX-015). In

addition, flow cytometry was performed to determine circulating levels of CD3, CD4, and CD8 cells at baseline.

ONYX-015

ONYX-015 (dl1520, also known as CI-1042) is a chimeric human group C adenovirus (Ad2 and Ad5) that does not express the 55-kd product of the *E1B* gene (Pfizer, Inc, Ann Arbor, MI, and Onyx Pharmaceuticals, Richmond, CA).³⁵ It contains a deletion between nucleotides 2,496 and 3,323 in the *E1B* region encoding the 55-kd protein. In addition, a C-to-T transition at position 2,022 in *E1B* generates a stop codon at the third codon position of the protein. These alterations eliminate expression of the *E1B*-55-kd gene in ONYX-015-infected cells. ONYX-015 was grown and titered on the human embryonic kidney cell line HEK293.

Detection of ONYX-015 Adenovirus

The TaqMan assay is designed to amplify an amplicon of 92 nts (nts 2,453 to 2,544) that is specific for ONYX-015 (Fig 1). The specific detection of ONYX-015 is due to two factors: the amplicon overlaps the *E1B* region deletion (911 nts are missing from the wild-type sequence) and an 8-base pair *Puc*-derived linker insert is part of the TaqMan probe. The lower limit of quantitation for the assay is 4.2×10^4 particles of ONYX-015 per mL of plasma. The lower limit of detection is 1.05×10^4 particles of ONYX-015 per mL of plasma. This assay is specific for ONYX-015 DNA and does not detect wild-type adenovirus sequences. Polymerase chain reaction (PCR) cycling conditions are as follows: hold at -50°C , 2 minutes; hold at -95°C , 10 minutes; 40 cycles at -95°C , 15 seconds; and -63°C . The presence of PCR inhibitors in the sample is monitored using an independent PCR reaction.

Patient samples are spiked with exogenous DNA to monitor recovery in the extraction step and the presence of PCR inhibitors. A standard curve is prepared by serial diluting ONYX-015 virus from 2×10^9 to 1.05×10^4 vp/mL. Negative controls consist of a plasma control without virus and a type D adenovirus wild-type control. Viral DNA is extracted from patient samples, standard, and controls using a QIAamp DNA mini kit (Valencia, CA). The amount of ONYX-015 viral DNA is then quantitated by reverse transcription PCR using the above-described specific primer and probe.

ONYX-015 Handling and Processing

ONYX-015 is formulated as a sterile viral solution in Tris buffer (Tris 10 mmol/L [pH 7.4], MgCl_2 1 mmol/L, CaCl_2 150 mmol/L, and 10% glycerol). The solution is supplied frozen (-20°C) in single-use, plastic screw-cap vials. Each vial contains 0.5 mL of virus solution at a specified viral titer. Vialled virus solution was thawed and diluted to the appropriate titer for dosing and was then further diluted to a final volume equivalent to 30% of the volume of the tumor to be injected. All dilutions were made with D5W (Baxter D5W electrolyte no. 45). Tumor volume was estimated by taking the product of the maximal

Fig 1. Schematic of the ONYX-015 detection amplicon. Nucleotides 2,453 to 2,544 of ONYX-015 are shown (5' strand only). This is the amplicon amplified in the TaqMan assay. The capital letters represent the sequence of the *Puc*-derived insert in construction of this virus. The underlined regions correspond to the 5' primer, the TaqMan probe, and the 3' primer. The probe and the 3' primer are homologous to the 3' strand.

tumor diameter, its perpendicular and estimated depth, and dividing by 2. Vials of ONYX-015 were opened and diluted immediately before injection in biologic safety cabinets at the patient treatment area. All waste items were disposed in biohazard containers and autoclaved or incinerated.

Treatment Regimen

To ensure uniform dosing to the injected tumor in each patient, a single tumor was identified for ONYX-015 injection in each patient. If more than one injectable tumor was present, the most symptomatic and/or largest tumor mass was injected with ONYX-015. The tumor was mapped into five equally spaced and equally sized sections. Local anesthesia was applied to the skin as needed. The tumor was injected once a day (standard schedule) or twice a day (hyperfractionated schedule) with 10^{10} particles into each of the five quadrants. The suspension volume of D5W saline used for ONYX-015 administration was normalized to 30% of the estimated volume of the tumor mass to be injected (see above). During each treatment session, one puncture of the skin was made at a site approximately 80% of the distance from the tumor center out to the tumor periphery. Six to eight needle tracts were made radially out from the puncture site; virus was administered equally along the length of the needle tracts (25-gauge needle). This approach was carried out each day from puncture sites that were equally spaced out and encompassed the entire tumor mass. The majority of the viral dose was administered at the tumor periphery and at the interface between normal tissue and tumor tissue.

In the initial phase of the study, tumor injections were performed once daily for 5 consecutive days (standard schedule); these injections were repeated every 3 weeks or until tumor progression. After documentation of safety with this regimen ($n = 30$), a more aggressive injection regimen was tested in a subsequent 10 patients by administering a four-fold higher dose; identical injections were performed twice daily for 5 days during each of the first 2 weeks on study (hyperfractionated schedule). After a 1-week rest period, the hyperfractionated regimen was repeated. Patients' vital signs were taken 15 minutes before and after each treatment for a minimum of 30 minutes. Patients were eligible for repeat treatment cycles at the same dosage every 3 weeks if no grade 4 toxicity with the prior treatment cycle of ONYX-015 occurred and no progression of the injected tumor was observed. After this induction regimen, maintenance treatment cycles were given by the same schedule as was used in the initial patient cohort (every 3 weeks as described above). The injections were given in outpatient clinics, including Mary Crowley Medical Research Center at Baylor University Medical Center (Dallas, TX), Albany Regional Cancer Center (Albany, NY), US Oncology Research (Houston, TX), Beatson Oncology Centre (Glasgow, Scotland), Dana-Farber Cancer Institute (Boston, MA), University of Chicago (Chicago, IL), and M.D. Anderson Cancer Center (Houston, TX).

Tumor Assessments

Tumor masses were measured serially by either physical examination or radiographic scanning (computed tomography or magnetic resonance imaging), whichever the principal investigator deemed most accurate for the measurement of the injected tumor mass. In general, very superficial lesions were measured by physical examination and deeper tumors were measured most accurately by radiographic scanning. Tumor measurements were determined either every 3 weeks (physical examination) or every 6 weeks (computed tomography/magnetic resonance imaging scans) while patients were on active study treatment; after treatment completion, patients' tumors were assessed

every 8 weeks or sooner if signs or symptoms of progression became evident. Radiographic scans were assessed by independent radiologists who were not investigators on the study.

The degree of necrosis induction within injected tumors was categorized as follows: complete regression, complete disappearance of measurable tumor; partial regression, $\geq 50\%$ but less than 100% decrease in nonnecrotic cross-sectional tumor area; minor response, less than 50%, $\geq 25\%$ decrease in nonnecrotic tumor area; stable disease, less than 25% decrease and less than 25% increase in nonnecrotic tumor area; progressive disease, $\geq 25\%$ increase in tumor area versus the baseline area. Radiologists were blinded to the *p53* gene status and neutralizing antibody titer of the patients at the time of tumor assessment. Tumors were considered assessable for response at earliest assessment at any time after the first injection. All lesions (injected/noninjected) were followed to assess response.

p53 Gene Sequencing Determination

Exons 5 through 9 of the tumor *p53* gene were sequenced completely during the first two thirds of the trial. Exons 2 through 11 were assessed by *p53* gene chip technology during the final one third of the trial. Since certain gene deletions can be missed by gene chip analysis (ie, a wild-type sequence is reported despite a functionally significant mutation), wild-type *p53* gene sequences by gene chip analysis underwent confirmatory sequencing to be validated.

Determination of Neutralizing Antibody Titers

Patient and control samples were incubated at 55°C for 30 minutes to inactivate complement. Clinical plasma samples previously determined to produce high, mid-range, and negative titers were designated as plasma controls. Each dilution was mixed with adenovirus stock at a titer prequalified to produce 15 to 20 plaques per well of a 12-well dish in DMEM growth medium. The patient samples and controls were inoculated for 1 hour at room temperature and applied to 70% to 80% confluent JH293 cells in 12-well dishes. After 2 hours of incubation at 37°C, 5% CO₂, plasma-virus mix was removed and 2 mL of 1.5% agarose in DMEM was added to each well. Plates were read on day 7 after inoculation by counting the number of plaques per well. The titer of neutralizing antibody for each sample was reported as the dilution of plasma that reduced the number of plaques to 60% of the number of plaques in the virus control without antibody. Determinations of neutralizing antibody titers were made before cycle 1 (baseline), before cycle 2, and before cycle 3.

Additional Follow-Up After Treatment Initiation

Neutralizing antibody titers were repeated every 4 weeks. Routine blood testing, including complete blood count and differential, electrolytes, blood urea nitrogen, creatinine, and liver function tests, was repeated every 3 weeks. Blood samples to determine circulating ONYX-015 after intratumoral injection at cycle 1 were determined on days 1 and 5.

RESULTS

Treatment

Forty patients were entered onto the trial from six sites (Mary Crowley Medical Research Center at Baylor University, M.D. Anderson Cancer Center, Beatson Cancer Institute, Albany Regional Cancer Center, Dana-Farber Cancer

Table 1. Patient Demographics

	ONYX-015 Standard Schedule (qd x 5/21-day cycle)		ONYX-015 Hyperfractionated Schedule (bid x 10/21-day cycle)	
	No. of Patients	%	No. of Patients	%
No. of patients registered	30		10	
Age, years				
Median	58		70	
Range	39-74		49-78	
Sex				
Male	23	77	8	80
Female	7	23	2	20
KPS score				
90-100	16	54	2	20
70-80	14	46	8	80
Prior therapy				
Surgery	21	70	7	70
Radiotherapy	28	93	10	100
Chemotherapy	19	63	6	60
≥ 2 modalities	27	90	8	80
Location of recurrence*				
Larynx	4	13	2	20
Tongue	2	7	0	0
Cervical area	17	57	1	10
Other	7	23	7	70
Tumor size, cm				
Maximum area				
Median	12.76		10.99	
Range	1.56-38.50		1.1-69	
Maximum diameter				
Median	3.4		3.85	
Range	1-8.4		0.9-7	
Baseline neutralizing < 1:20	14	47	2	22
Antibody levels ≥ 1:20	16	53	7	78
Baseline CD4 counts				
Median	334.22		294	
Range	79.24-1,318.05		188-798	
p53 gene status				
Mutant	12	40	6	60
Wild-type	11	37	2	20
Undetermined	7	23	2	20

Abbreviation: KPS, Karnofsky performance status.

*Two patients had distant pulmonary metastases that were not injected.

Institute, the University of Chicago) between July 1997 and September 1998. The first 30 patients were enrolled onto the standard ONYX-015 schedule trial; the 10 patients enrolled subsequently received the hyperfractionated regimen. All patients registered received at least a single injection of ONYX-015 and were assessable for toxicity. Thirty-six patients were considered assessable for initial response. Two patients (one standard, one hyperfractionated) were not assessable due to death before response assessment (not treatment-related), and two patients (hyperfractionated) withdrew before response assessment. Characteristics of patients receiving the standard versus hyperfractionated schedule are listed in Table 1. As listed in Table 2, 70 cycles

(345 doses) were administered to 30 patients who received standard-schedule ONYX-015, and 27 cycles (188 doses) were administered to 10 patients who received the hyperfractionated schedule.

Tumor Response

Data on the response of injected tumors is listed in Table 3. Four patients (14%) who received the standard dosing schedule achieved a partial or complete regression of the injected tumor, 12 (41%) had stable disease, and 13 (45%) progressed. One (14%) of the hyperfractionated patients achieved a complete regression, four (58%) achieved stable disease, and two (29%) progressed. The median time to

Table 2. Treatment Parameters

	ONYX-015 Standard Schedule	ONYX-015 Hyperfractionated Schedule
No. of cycles per patient		
Median	2	2
Range	1-8	1-6
Total number of cycles	70	27
No. of injections	325	188
No. of days on study per patient		
Median	35.5	87.5
Range	13-175	40-254
Dilution volume, ml per patient per injection		
Median	6.05	5.85
Range	0.1-53.1	0.1-42.4

involved tumor progression, progression-free survival, and survival with the standard versus hyperfractionated approaches are listed in Table 3. No significant differences were observed between the two dosing regimens. There was no correlation between baseline tumor area, neutralizing antibody level, and response. A significant correlation was demonstrated between antitumoral activity (complete, partial, and minor responses) and presence of a *p53* gene mutation ($P = .017$).

Toxicity

Toxicity that occurred in more than 25% of patients is listed in Table 4. The majority of the toxic effects were of

mild or moderate intensity. Fatal toxicity not related to ONYX-015 occurred in three standard-treatment patients (10%) and one hyperfractionated-treated patient (10%). One fatality was related to hematemesis from an unrelated gastrointestinal ulcer, one was due to hemorrhage from local progression, one was due to bacterial-induced septic shock, and one was due to anoxia caused by airway obstruction from progressive disease. Fourteen serious adverse events were reported in the standard arm, and nine serious adverse events occurred in the hyperfractionated arm. One serious adverse event was categorized as "probably related" to study medication in the standard arm (hemorrhage at injection site). The following events were categorized as possibly related to ONYX-015 injection: pneumonia with no organisms identified ($n = 1$), confusion (although concurrent hypocalcemia may have been related; $n = 1$), and recurrent atrial flutter ($n = 1$). The other 10 serious adverse events were either not related or the relationships were unable to be determined. Among patients who received hyperfractionated treatment, one patient developed injection site hemorrhage categorized as possibly related to ONYX-015 injection. Other events were categorized as not related or unable to be determined. Nine of 40 patients developed pneumonia not related to study treatment (six standard and three hyperfractionated). A specific cause of pneumonia was identified in three patients (two bacterial and one unrelated peptic ulcer perforation). The six other causes were thought to be related to aspiration associated with the cancer. The pneumonia lasted from 6 to 13 days. Six patients were retreated with ONYX-015 after resolution of pneumonia without recurrence. Reasons for study discontinuation are listed in Table 5.

Systemic Distribution of ONYX-015

All 30 patients (29 of 30 in cycle 1) in the standard arm were tested for circulating ONYX-015 using PCR analysis 24 hours after the 5-day intratumoral injection series. Previous studies have shown rapid clearance of the ONYX-015 genome from the blood (approximately 6 hours); therefore, viremia ≥ 24 hours after the last injection is strong evidence for viral replication and shedding. Detectable levels of ONYX-015 were identified in 12 (41%) of 29 patients 24 hours after the last ONYX-015 injection (Table 6). In two patients (9%), the ONYX-015 genome was detected 10 days after injection in cycle 1. No samples were positive for circulating ONYX-015 genome 22 days after any injection in cycle 1 or any other cycle, and 15 days after any injection beyond cycle 1. Six (28%) of 21 patients had detectable circulating ONYX-015 genome in cycle 2 24 hours after intratumoral injection, and two of eight patients had detectable circulating ONYX-015 genome 24 hours

Table 3. Response of Injected Lesions

	ONYX-015 Standard	ONYX-015 Hyperfractionated
Complete response	2 (7)	1*
Partial response	2 (7)	0
Minor response	2 (7)	1
Stable disease	10 (34)	3
Progressive disease	13 (45)	2
Not assessable	1†	3‡,§
Median progression-free survival, days:		
Responders (PR, CR, MR, SD)	59	80
Nonresponders	21	16.5
Nonassessable	—	5
All	22	53
Median survival, days:		
Responders (PR, CR)	125	152
Nonresponders	183	58
Nonassessable	25	—
All	126	75

Abbreviations: PR, partial response; CR, complete response; MR, minor response; SD, stable disease.

*Complete histologic response at autopsy.

†Death not related to treatment occurred before tumor assessment.

‡Patient withdrew before response assessment.

§Derived by the Kaplan-Meier product-limit method.

Table 4. Toxicity Occurring in More Than 25% of Patients Possibly Related to Study Treatment

Toxicity	Grade						Total	
	Mild	Moderate	Severe	Life Threatening	Fatal*	Unknown	No.	%
Standard approach (n = 30)								
Fever	9	12	0	0	0	1	22	73
Asthenia	5	5	5	0	0	0	15	50
Chills	7	6	2	0	0	0	15	50
Injection site pain	1	7	6	0	0	0	14	47
Headache	1	5	3	0	0	0	9	30
Nausea	1	4	4	0	0	0	9	30
Dyspnea	0	6	2	0	0	0	8	27
Hyperfractionated approach (n = 10)								
Fever	1	4	1	0	0	0	6	60
Injection site pain	6	2	0	0	0	0	8	80
Asthenia	1	4	1	0	0	0	6	60
Headache	1	2	0	0	0	0	3	30
Bacterial infection	1	2	0	0	0	0	3	30
Depression	2	1	0	0	0	0	3	30
Respiratory disorder	1	0	2	1	0	0	4	40
Pneumonia	0	2	1	0	0	0	3	30
Nausea	3	3	1	0	0	0	7	70
Anorexia	3	0	0	0	0	0	3	30
Hypotension	0	1	1	2	0	0	4	40
Hemorrhage	1	0	1	1	0	0	3	30
Tachycardia	2	0	1	0	0	0	3	30
Hyponatremia	0	3	0	1	0	0	4	40
Sweating	2	1	0	0	0	0	3	30
Anemia	0	1	2	0	0	0	3	30

*In the standard approach group, death that occurred in three patients was related to hematoma, hemorrhage, and bacterial sepsis and was not related to study medication. Among the patients treated with the hyperfractionated schedule, death occurred in one patient due to bronchial obstruction induced by progressive disease.

after injection in cycle 3. The two patients who had detectable ONYX-015 genome in cycle 3 achieved a minor response and a complete response. Otherwise no correlation between circulating genome and response was observed in patients with detectable genome in cycle 2 or patients with circulating genome in cycle 1. Patients entered onto the hyperfractionated treatment arm were not followed for systemic distribution of ONYX-015 genome.

Table 5. Reasons for Study Discontinuation

	Standard		Hyperfractionated	
	No. of Patients	%	No. of Patients	%
Disease progression at injected site	10	33	1	10
Disease progression at noninjected site	10	33	4	40
Patient decision	4	13	1	10
Unrelated medical condition(s)	1	3	0	0
Investigator's decision	1	3	2	20
Death	3	10	1	10
Other	1	3	1	10

Neutralizing Antibody Titers

Sixteen patients who received standard ONYX-015 and seven patients who received hyperfractionated ONYX-015 were identified as having high (elevated > 1:20) neutralizing antibody titers at baseline (Table 1). Fifty-three percent

Table 6. PCR-Detectable ONYX-015 Genome in Circulation After Intratumoral Injection

Cycle	Days 6 and 7		Day 15		Day 22	
	+	-	+	-	+	-
1	12	17	2	19	0	22
2	6	15	0	17	0	18
3	2	6	0	7	0	6
4	0	2	0	2	0	2
5	0	1	0	1	0	0
6	0	1	0	0	0	0
7	1	0	0	1	0	1
8	0	1	0	1	0	1

Symbols: +, number of patients with positive detectable genome; -, number of patients with genome not detectable.

of standard-arm patients had antibody titers more than 1:20 at baseline, and 23 (96%) of 24 patients measured after cycle 1 had antibody titers above 1:20. All patients in the standard arm had neutralizing antibody titers above 1:20 after cycle 2. The median antibody titers at baseline ($n = 30$) was 51 (range, 0 to 1,798). After cycle 1 ($n = 24$), the median titer was 11,896 (range, 0 to 81,920). After cycle 2 ($n = 14$), the median titer was 12,363 (range, 225 to 71,425). Similar titers were seen in the hyperfractionation-treated patients. At baseline ($n = 9$), the median neutralizing titer was 1,074 (range, 0 to 8,847). This increased to 9,733 (range, 2,165 to 62,700) after cycle 2 ($n = 5$). There was no correlation of baseline titer levels to tumor response, time to local progression, progression-free survival duration, or overall survival.

DISCUSSION

The results from these trials indicate that intratumoral injection of the replication-selective adenovirus ONYX-015 at a dose of 1×10^{10} pfu daily for 5 days of a 21-day cycle was well tolerated. Transient low-grade fever and injection site pain were the most frequent toxicities. These were manageable on an outpatient basis. Antitumor activity (as measured by $\geq 50\%$ tumor destruction) was observed in approximately 14% of patients and did not seem different between the standard and hyperfractionation arms. Survival was also not different between the two arms; however, injection site pain occurred more frequently on the hyperfractionated regimen. Future proof of clinical benefit will be necessary to determine clinical utility. These data suggest that ONYX-015 has a favorable safety profile and modest efficacy in recurrent head and neck cancer as a single agent. Future testing in this patient population has, therefore, focused on combinations with standard agents, such as cisplatin-based chemotherapy.⁴³

Replication-competent viruses have been tested as therapeutic agents for more than 100 years. Smallpox was eradicated with a replicating virus vaccine.^{44,45} Exploration of the use of replicating viruses for the treatment of cancer was documented as early as 1912 when a woman with advanced cervical cancer achieved a response after injection with an attenuated rabies virus.^{46,47} In 1950, the oncolytic activity of Egypt 101 virus was validated in vitro,⁴⁸ and clinical activity was suggested after intratumoral injection in cancer patients.⁴⁹⁻⁵¹ However, the antitumoral effects were transient (< 3 months). Subsequent clinical investigation with mumps virus as a cancer therapy was reportedly associated with a 41% "response" rate in 90 treated patients.⁵² However, a follow-up trial⁵³ involving 200 cancer patients in whom mumps virus was administered by a multiple intratumoral injection schedule revealed transient

tumor regression in only 26 patients. Toxicity was limited to transient fever and injection site pain. Another oncolytic virus, Newcastle disease virus (NDV),⁵⁴⁻⁵⁸ showed selective replicative capacity in malignant cells. The mechanism of NDV selectivity may be related to elevated *myc* oncogene expression or differences in membrane permeability, as opposed to the *E1B-55-kd* deletion effect on *p53* with ONYX-015.⁵⁸⁻⁶⁰ Additionally, consistent with what we observed with ONYX-015, tumor response was correlated with viral replication-induced oncolysis.⁵⁷ NDV was used to lyse tumor cells in vitro for the purpose of creating a viral oncolysate (virus and lysed tumor cells). Several trials in melanoma patients with limited-stage disease undergoing surgical resection followed by vaccination with the NDV viral oncolysate suggested improved survival compared with historical controls.⁶¹⁻⁶⁴ Similar results have been found in separate trials involving patients with colorectal carcinoma,⁶⁵ advanced renal cell carcinoma,⁶⁶ metastatic breast cancer, and ovarian cancer.⁶⁷ Influenza virus and vaccinia virus have also been studied as a viral oncolysate for tumor vaccine trials.⁶⁸⁻⁷⁰ More recently, a variety of replication-selective viruses have been either engineered for replication selection (including human adenovirus, herpes virus, and vaccinia virus)⁷¹ or shown to be replication-selective based on specific genetic tumor target (ie, activated *ras* for retrovirus).⁷²⁻⁷⁵ Replication-selective, tumor-targeting bacteria such as *Salmonella typhimurium* have also shown encouraging preclinical activity.

A great deal of data have been accumulated suggesting that adenovirus serotype 5 is an effective oncolytic virus with a low toxicity profile to humans. DNA from thousands of human tumors have been analyzed for the presence of adenovirus DNA, and no integrated viral DNA has been isolated from any human tumor.⁷² Eighty percent of adults have existing antibodies to adenovirus serotype 5, but less than 15% of exposed patients become clinically symptomatic.⁷³ The most common symptoms of an adenoviral serotype 5 infection are flu-like in nature and include cough, gastroenteritis, conjunctivitis, and, rarely, pneumonia. However, these symptoms are rarely seen even in immunocompromised patients.⁷⁴ Oral adenoviral vaccines were given to thousands of military recruits in the 1960s without adverse effects or increase in cancer.⁷⁵ Long- and short-term safety of intratumoral adenoviral injection has been shown in several animal cancer models,⁷⁶⁻⁸² and live adenovirus inocula were given intratumorally and intra-arterially to patients with cervical carcinoma at the National Cancer Institute in the 1950s.⁵¹ Again, no significant toxicities, other than transient fever and malaise, were observed, even in subsets of patients treated with corticosteroids and in those in whom neutralizing adenovirus antibodies were not

present. Intravascular administration was also well tolerated in a small group of patients.⁸³ Adenoviral vectors with the E1 and E3 deletion containing the *Escherichia coli* cytosine deaminase gene have also been administered via intradermal injection to normal individuals in studies of toxicity and immune response at dose levels of 10^6 , 10^7 , and 10^8 pfu.⁸⁴ No significant toxicity was observed.⁸⁴ This was consistent with clinical trial results in the same patient population of head and neck cancer patients described in this trial who received a nonreplicating adenoviral vector containing a wild-type *p53* gene.^{85,86}

Given the safety and toxicity profile of ONYX-015, it seems reasonable to explore this virus in patients with earlier-stage disease⁸⁷⁻⁹⁰ and possibly even to enhance sensitivity when combined with chemotherapy or radiation therapy.³⁹ Independent of the ONYX-015 replication-induced oncolysis, ONYX-015 *E1A* gene expression can activate the cell cycle and increase cellular sensitivity to chemotherapy or radiation therapy.

Use of ONYX-015 for local management of SCCHN and as adjuvant therapy after surgical resection of SCCHN and, possibly, other malignant tumors should also be considered but will require further investigation. Comparison of survival between responding and nonresponding patients will also need to be followed in the future, although differences observed in this trial were not significant. Pursuit of other schedules of intratumoral administration (ie, > 5 days/21-

day course) are unlikely to be of value, although justification of a systemic infusion schedule for ONYX-015 may be warranted since it has been shown to be safe and efficacious in animal cancer models.³⁹ Detection of ONYX-015 genome in plasma on the last day of ONYX-015 injection suggests that circulating virus, at low plasma concentration, is safe. Furthermore, persistent detection in two patients 10 days after the last injection suggests that a viral replicative process was ongoing, although it did not persist since none of the samples tested showed evidence of circulating viral genome more than 17 days after the last injection.

Future work with ONYX-015 and other replication-selective viruses will also explore the possibility of arming these viruses with exogenous genes, particularly if selective tumor replication is confirmed. Antitumor effects correlating with enhanced cytotoxic T-lymphocyte activity have been noted in vivo with replication-selective herpes simplex virus (G207) carrying an interleukin 12 gene,⁷¹ for example. Over the next year, a number of these replication-selective agents are expected to enter clinical testing.

ACKNOWLEDGMENT

The authors thank Ana Petrovich for manuscript preparation, Angela Buchanan for analysis interpretation, Sherry Toney for extensive time and effort in coordinating study samples and results as well as editorial proofing of the manuscript, and Carrie LeDuc from Althea for her assistance with sequence interpretation and for providing the methods discussion for sequencing.

REFERENCES

- Vokes EE, Weichselbaum RR, Lippman SM, et al: Head and neck cancer. *N Engl J Med* 328:184-194, 1993
- Lippman SM, Vokes EE: Complications of chemotherapy, in Cline LG, Larson DL, Shah JP (eds): *Essentials of Head and Neck Oncology*. Thieme Medical Publishers Inc, 1998, pp 408-415
- Forastiere AA, Metc B, Schaller DE, et al: Randomized comparison of cisplatin plus fluorouracil and carboplatin plus fluorouracil versus methotrexate in advanced squamous cell carcinoma of the head and neck: A Southwest Oncology Group study. *J Clin Oncol* 10:1245-1251, 1992
- Jacobs C, Lyman G, Velez-Garcia E, et al: A phase III randomized study comparing cisplatin and fluorouracil as single agents and in combination for advanced squamous cell carcinoma of the head and neck. *J Clin Oncol* 10:257-263, 1992
- Paredes J, Hong WK, Felder TB, et al: Prospective randomized trial of high-dose cisplatin and fluorouracil infusion with or without sodium diethyldithiocarbamate in recurrent and/or metastatic squamous cell carcinoma of the head and neck. *J Clin Oncol* 6:955-962, 1988
- Clavel M, Vermorken JB, Cognetti P, et al: Randomized comparison of cisplatin, methotrexate, bleomycin and vincristine (CABO) vs. cisplatin and 5-FU vs. cisplatin in recurrent or metastatic squamous cell carcinoma of the head and neck: A phase III study of the EORTC Head and Neck Cancer Cooperative Group. *Ann Oncol* 5:521-526, 1994
- Schrijver D, Johnson J, Jimenez U, et al: Phase III trial of modulation of cisplatin/fluorouracil chemotherapy by IFN- α -2b in patients with recurrent or metastatic head and neck cancer: Head and Neck Interferon Cooperative Study Group. *J Clin Oncol* 16:1054-1059, 1998
- Group LHaNO: A phase III randomized trial of cisplatin, methotrexate, cisplatin + methotrexate and cisplatin + 5-FU in end stage squamous carcinoma of the head and neck. *Br J Cancer* 61:311-315, 1990
- Kish JA, Ensley JF, Jacobs JR, et al: Evaluation of high dose cisplatin and 5-FU infusion as initial therapy in advanced head and neck cancer. *Am J Clin Oncol* 11:553-557, 1988
- Buesa JM, Fernandes R, Esteban E, et al: Phase II trial of ifosfamide in recurrent and metastatic head and neck cancer. *Ann Oncol* 2:151-152, 1991
- Huber MH, Lippman SM, Benner SE, et al: A phase II study of ifosfamide in recurrent squamous cell carcinoma of the head and neck. *Am J Clin Oncol* 19:379-382, 1996
- Martin M, Diaz-Rubio E, Gonzales-Larriba JL, et al: Ifosfamide in advanced epidermoid head and neck cancer. *Cancer Chemother Pharmacol* 31:340-342, 1993
- Pai VR, Parikh DM, Mazumdar AT, et al: Phase II study of high dose ifosfamide as a single agent in combination with cisplatin in the treatment of advanced and/or recurrent squamous cell carcinoma of head and neck. *Oncology* 50:85-91, 1993

14. Shin DM, Glisson BS, Khuri FR, et al: Phase II trial of paclitaxel, ifosfamide, and cisplatin in patients with recurrent head and neck squamous cell carcinoma. *J Clin Oncol* 16:1325-1330, 1998
15. Forastiere AA, Shank D, Neuberg D, et al: Final report of a phase II evaluation of paclitaxel in advanced squamous cell carcinoma of the head and neck: An Eastern Cooperative Oncology Group trial (PA390). *Cancer* 82:2270-2274, 1998
16. Smith RE, Thornton DE, Allen J: A phase II trial of paclitaxel in squamous cell carcinoma of the head and neck with correlative laboratory studies. *Semin Oncol* 22:41-46, 1995
17. Forastiere AA, Leong T, Murphy B, et al: A phase III trial of high dose paclitaxel + cisplatin + G-CSF vs. low dose paclitaxel + cisplatin in patients with advanced squamous cell carcinoma of the head and neck: An Eastern Cooperative Oncology Group trial. *Proc Am Soc Clin Oncol* 16:384a, 1997 (abstr 1367)
18. Benner SE, Lippman SM, Huber MH, et al: Phase I study of paclitaxel, cisplatin and ifosfamide in patients with recurrent or metastatic squamous cell carcinoma of the head and neck. *Semin Oncol* 22:22-25, 1995
19. Catimel G, Verweij J, Mattijssen V, et al: Docetaxel (Taxotere): An active drug for the treatment of patients with advanced squamous cell carcinoma of the head and neck. *Ann Oncol* 5:533-537, 1994
20. Dreyfuss AI, Clark JR, Norris CM, et al: Docetaxel: An active drug for squamous cell carcinoma of the head and neck. *J Clin Oncol* 5:533-537, 1994
21. Ebihara S, Fujii H, Sasaki Y, et al: A late phase II study of docetaxel (Taxotere) in patients with head and neck cancer. *Proc Am Soc Clin Oncol* 16:399a, 1997 (abstr 1425)
22. Schoffski P, Wanders J, Catimel G, et al: A promising regimen for treatment of squamous cell carcinoma of the head and neck: Docetaxel and cisplatin. *Ann Oncol* 7:79, 1996 (abstr 373P)
23. Oliveira J, Geoffrois L, Rolland F, et al: Phase II study of Navelbine in patients with metastatic and/or local recurrent squamous cell carcinoma of the head and neck untreated by chemotherapy and with lesions with previously irradiated fields. *Ann Oncol* 7:78, 1996 (abstr 3710)
24. Oliveira J, Geoffrois L, Rolland F, et al: Activity of Navelbine on lesions within previously irradiated fields in patients with metastatic and/or local recurrent squamous cell carcinoma of the head and neck: An EORTC-ESCG study. *Proc Am Soc Clin Oncol* 16:406a, 1997 (abstr 1449)
25. Canfield VA, Saxman SB, Kolodziej MA, et al: Phase II trial of vinorelbine in advanced or recurrent squamous cell carcinoma of the head and neck. *Proc Am Soc Clin Oncol* 16:387a, 1997 (abstr 1382)
26. Degardin M, Bastin PH, Rolland F, et al: Phase II study of vinorelbine in patients with metastatic and/or recurrent squamous cell carcinoma of the head and neck. *Eur J Cancer* 33:187, 1997 (abstr 843)
27. Smith RE, Lew D, Rodriguez GI, et al: Evaluation of topotecan in patients with recurrent or metastatic squamous cell carcinoma of the head and neck: A phase II Southwest Oncology Group study. *Invest New Drugs* 14:403-407, 1996
28. Robert F, Soong SJ, Wheeler RH: A phase II study of topotecan in patients with recurrent head and neck cancer: Identification of an active new agent. *Am J Clin Oncol* 20:298-302, 1997
29. Catimel G, Vermorken JB, Clavel M, et al: A phase II study of gemcitabine in patients with advanced squamous cell carcinoma of the head and neck. *Ann Oncol* 5:543-547, 1994
30. Merlano M, Benasso Macorvo R, et al: Gemcitabine, cisplatin and radiotherapy in squamous cell carcinoma of the head and neck. *Proc Am Soc Clin Oncol* pp:405a, 1997 (abstr 1445)
31. Fountzilas G, Athanassiades A, Kalogera-Fountzila A, et al: Paclitaxel in combination with carboplatin or gemcitabine for the treatment of advanced head and neck cancer. *Semin Oncol* 24:28-32, 1997
32. Rowland KMJ, Taylor SGT, Spiers AS, et al: Cisplatin and 5-FU infusion chemotherapy in advanced, recurrent cancer of the head and neck: An Eastern Cooperative Oncology Group study. *Cancer Treat Rep* 70:461-464, 1986
33. Rooney M, Kish J, Jacobs J, et al: Improved complete response rate and survival in advanced head and neck cancer after 3-course induction therapy with 120-hour 5-FU infusion and cisplatin. *Cancer* 55:1123-1128, 1985
34. Bischoff JR, Kim DH, William A, et al: An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. *Science* 274:373-376, 1996
35. Lechner MS, Mack DH, Finicle AB, et al: Human papilloma virus E6 proteins bind p53 in vivo and abrogate p53-mediated repression of transcription. *EMBO J* 11:3045-3052, 1992
36. Gannon JV, Lane DP: P53 and DNA polymerase alpha compete for binding to SV40 T antigen. *Nature* 329:456-458, 1987
37. Barker DD, Berk AJ: Adenovirus proteins from both E1B reading frames are required for transformation of rodent cells by viral infection and DNA transfection. *Virology* 156:107-121, 1987
38. Neda H, Wu CH, Wu GY: Chemical modification of an ecotropic murine leukemia virus results in redirection of its target cell specification. *J Biol Chem* 266:14143-14146, 1991
39. Heise C, Sampson-Johnes A, Williams A, et al: ONYX-015, an E1B gene-attenuated adenovirus, causes tumor-specific cytotoxic antitumor efficacy that can be augmented by standard chemotherapy agents. *Nat Med* 3:639-645, 1997
40. Goodrum FD, Omelles D: P53 status does not determine outcome of E1B 55-kilodalton mutant adenovirus lytic infection. *J Virol* 72:9479-9490, 1998
41. Hall AR, Dix BR, O'Carroll SJ, et al: P-53-dependent cell death/apoptosis is required for a productive adenovirus infection. *Nat Med* 4:1068-1072, 1998
42. Kim D, Hermiston T, McCormick LF: ONYX-015: Clinical data are encouraging. *Nat Med* 4:1341-1342, 1998
43. Kim D, Khuri F, Nemunaitis J, et al: A phase II trial of ONYX-015, a selectively replicating adenovirus, in combination with cisplatin and 5-FU in patients with recurrent head and neck cancer. *Proc Am Soc Clin Oncol* 18:389a, 1999 (abstr 1505)
44. Niemialowski MG, Toka FN, Malicka E, et al: Controlling orthopox virus infections: 200 years after Jenner's revolutionary immunization. *Arch Immunol Ther Exp* 44:373-378, 1996
45. Ellner PD: Smallpox: Gone but not forgotten. *Infection* 26:263-269, 1998
46. De Pace NG: *Ginnecologia* 9:82, 1912
47. Pack GT: Note of the experimental use of rabies vaccine for melanomatosis. *Arch Dermatol Syphilol* 62:694-695, 1950
48. Southam CM, Moore AE: Clinical studies of viruses as antineoplastic agents with particular reference to Egypt 101 virus. *Cancer* 5:1025-1034, 1952
49. Asada T: Treatment of human cancer with mumps virus. *Cancer* 34:1907-1928, 1974
50. Yamanishi E, Takahashi M, Kurimura T, et al: Studies on live mumps virus vaccine: III. Evaluation of newly developed live mumps virus vaccine. *Biken J* 13:157-161, 1970
51. Smith RR, Huebner JR, Rowe WP, et al: Studies on the use of viruses in the treatment of carcinoma of the cervix. *Cancer* 9:1211-1218, 1956

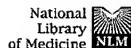
52. Moore AE: Carcinolytic viruses, in Harris RJC (ed): *Biological Approaches to Cancer Chemotherapy*. New York, NY, Academic Press, 1961, pp 365-370
53. Shimizu Y, Hasumi K, Okudaira Y: Immunotherapy of advanced gynecologic cancer patients utilizing mumps virus. *Cancer Detect Prev* 12:487-495, 1988
54. Flanagan AD, Love R, Tesar W: Propagation of Newcastle disease virus in Ehrlich ascites cells in vitro and in vivo. *Proc Soc Biol Med* 90:82-86, 1955
55. Prince AM, Ginsberg HS: Immunohistochemical studies on the interaction between Ehrlich ascites tumor cells and Newcastle disease virus. *J Exp Med* 105:177-187, 1957
56. Sinkovics J: Studies on the biological characteristics of the Newcastle disease virus (NDV) adapted to the brain of newborn mice. *Arch Ges Virusforsch* 7:403-411, 1957
57. Okuno Y, Asada T, Yamanishi K: Studies on the use of mumps virus for treatment of human cancer. *Biken J* 21:37-49, 1978
58. Cassel WA, Garrett RE: Newcastle disease virus as an antineoplastic agent. *Cancer* 18:663-668, 1965
59. Reichard KW, Lorence RM, Cascino CJ, et al: Newcastle disease virus selectively kills human tumor cells. *J Surg Res* 52:448-453, 1992
60. Sinkovics J, Howe CD: Super-infection of tumors with viruses. *Experientia* 25:733-734, 1969
61. Marsch M, Helenius A: Virus entry into animal cells. *Adv Virus Res* 36:107-151, 1989
62. Cassel WA, Murray DR: Letter to the Editor. *Nat Immun Cell Growth Regul* 7:351-352, 1988
63. Eilber FR, Morton LDL, Holmes CE, et al: Adjuvant immunotherapy with BCG in treatment of regional lymph node metastases from malignant melanoma. *N Engl J Med* 294:237-240, 1976
64. Gutterman JU, McBride C, Freireich EJ, et al: Active immunotherapy with BCG for recurrent malignant melanoma. *Lancet* 1:1208-1212, 1973
65. Schlag P, Manasterski M, Gerneth T, et al: Active specific immunotherapy with Newcastle disease virus modified autologous tumor cells following resection of live metastases in colorectal cancer: First evaluation of clinical response of a phase II trial. *Cancer Immunol Immunother* 35:325-330, 1992
66. Kirschner HH, Anton P, Atzpodien J: Adjuvant treatment of locally advanced renal cancer with autologous virus-modified tumor vaccines. *World J Urol* 13:171-173, 1995
67. Haas C, Straus G, Moldenhauer G, et al: Biospecific antibodies increase T-cell stimulatory capacity in vitro of human autologous virus-modified tumor vaccine. *Clin Cancer Res* 4:721-730, 1998
68. Reichard KW, Lorence RM, Cascino CJ: N-myc oncogene enhances the sensitivity of neuroblastoma to killing by Newcastle disease virus. *Surg Forum* 43:603-606, 1992
69. Boone CW: Augmented immunogenicity of tumor cell homogenates infected with influenza virus. *Recent Results Cancer Res* 47:394-400, 1974
70. Freedman RS, Edwards CL, Bowen JM, et al: Viral oncolysates in patients with advanced ovarian cancer. *Gynecol Oncol* 29:337-347, 1988
71. Toda M, Martuza RL, Kojima H, et al: In situ cancer vaccination: An IL-12 defective vector/replication-competent herpes simplex virus combination induces local and systemic antitumor activity. *J Immunol* 160:4457-4464, 1998
72. Green M, Wold WS, Mackey JK, et al: Analysis of human tonsil and cancer DNAs and RNAs for DNA sequences in group C (serotype 1, 2, 5 and 6) human adenoviruses. *Proc Natl Acad Sci U S A* 76:6606-6610, 1979
73. Brandt CD, Kim HW, Vargosko AJ, et al: Infections in 18,000 infants and children in controlled study of respiratory tract disease: Adenovirus pathogenicity in relation to serologic type and illness syndrome. *Am J Epidemiol* 90:484-500, 1969
74. Hierholzer JC: Adenoviruses in the immunocompromised host. *Clin Microbiol Rev* 5:262-274, 1992
75. Takafuji ET: Simultaneous administration of live, enteric-coated adenovirus types 4, 7 and 21 vaccines: Safety and immunogenicity. *J Infect Dis* 140:48-53, 1979
76. Lesoon-Wood LA, Kim WH, Kleinman HK: Systemic gene therapy with p53 reduces growth and metastases of a malignant human breast cancer in nude mice. *Hum Gene Ther* 6:395-405, 1995
77. Zhang W, Alemany R, Wang J: Safety evaluation of AdCMV-p53 in vitro and in vivo. *Hum Gene Ther* 6:155-164, 1995
78. Nielsen LL, Dell J, Maxwell E: Efficacy of p53 adenovirus-mediated gene therapy against human breast cancer xenografts. *Cancer Gene Ther* 4:129-138, 1997
79. Simon LRH, Engelhardt JF, Yang Y: Adenovirus-mediated transfer of the CFRT gene to lung of non-human primates: Toxicity study. *Hum Gene Ther* 4:771-780, 1993
80. Xu M, Kumar D, Srinivas S: Parenteral gene therapy with p53 inhibits human breast tumor in vivo through a bystander mechanism without evidence of toxicity. *Hum Gene Ther* 8:177-185, 1998
81. Gomez-Fox AM, Coats WS, Baque S: Adenovirus-mediated transfer of the muscle glycogen phosphorylase gene into hepatocytes confers altered regulation of glycogen. *J Biol Chem* 267:25129-25134, 1992
82. Le Gal La Salle G, Robert JJ, Bernard S: An adenovirus vector for gene transfer into neurons and glia in the brain. *Science* 259:988-990, 1993
83. Tursz T, Le Cesane A, Baldeyrou P: Phase I study of a recombinant adenovirus-mediated gene transfer in lung cancer patients. *J Natl Cancer Inst* 88:1857-1863, 1996
84. Harvey BG, Worgall S, Ramirez M: Host responses to intradermal administration of a first generation replication deficient adenovirus vector to normal individuals. *Proc Am Soc Gene Ther* p 43a, 1998 (abstr 167)
85. Nemunaitis J, Swisher G, Timmons T, et al: Adenovirus-mediated p53 gene transfer in sequence with cisplatin to tumors of patients with non-small-cell lung cancer. *J Clin Oncol* 18:609-622, 2000
86. Swisher SG, Roth JA, Nemunaitis J, et al: Adenoviral-mediated p53 gene transfer in advanced non-small cell lung cancer. *J Natl Cancer Inst* 91:763-771, 1999
87. You L, Yang C-T, Jablons DM: ONYX-015 works synergistically with chemotherapy in lung cancer cell lines and primary cultures freshly made from lung cancer patients. *Cancer Res* 60:1009-1013, 2000
88. Tainien M, Spitkovsky D, Jansen-Durr P, et al: Expression of E1A in terminally differentiated muscle cells reactivates the cell cycle and suppresses tissue-specific genes by separable mechanisms. *Mol Cell Biol* 16:5302-5312, 1996
89. Sanchez-Prieto R, Quintanilla M, Cano A, et al: Carcinoma cell lines become sensitive to DNA-damaging agents by the expression of the adenovirus E1A gene. *Oncogene* 13:1083-1092, 1996
90. Marchetti E, Romero J, Sanchez R, et al: Oncogene and cellular sensitivity to radiotherapy: A study on murine keratinocytes transformed by v-H-ras, v-myc, v-neu, adenovirus E1A and mutant p53. *Int J Oncol* 5:611-618, 1994

26, first paragraph]. Moreover, Roberts et al. disclose that their methods could be used to treat melanomas (see page 30, last paragraph).

Roberts et al. differ from instant invention in that they do not disclose the use of cells (i.e. producer cells) for the delivery of the oncolytic virus.

Coukos et al. disclose the use of producer cells for the delivery of oncolytic HSV-1 to tumor cells (see abstract). Moreover, Coukos et al. disclose that the use of producer cells may have many advantages over direct injection methods. Said advantages include: 1) amplification of viral load; 2) delivery of a virus within a producer cell may enable the virus to elude the subjects immune system; 3) use of producer cells with a binding affinity for the tumor cells would increase the localization of virus delivery; and 4) a vaccine antitumor response in selective patients might be generated (see page 1536).

Consequently, it would have been obvious to one of skill in the art to use producer cells, as disclosed by Coukos et al., in the method of melanoma tumor cell treatment disclosed by Roberts et al. One would have been motivated to do so in order to receive the benefits associated with the use of producer cells, as disclosed by Coukos et al. and cited above. One of ordinary skill in the art would necessarily have a reasonable expectation of success since both methods utilize oncolytic viruses to treat tumor cells. Moreover, given the success of using carrier cells to deliver oncolytic HAV-1, it would have been obvious for the skilled artisan to try and adapt said system to other oncolytic virus (e.g. VSV) types. Finally, given that the use of VSV as a cancer treatment is well known in the art yielding predictable results, it is obvious for the skilled artisan to use the VSV of Roberts et al. (see *KSR International Co. v. Teleflex Inc.*, No. 04-1350 [U.S. Apr. 30, 2007]).



[All Databases](#)
[PubMed](#)
[Nucleotide](#)
[Protein](#)
[Genome](#)
[Structure](#)
[OMIM](#)
[PMC](#)
[Journals](#)
[Book](#)

Search for

[Limits](#)
[Preview/Index](#)
[History](#)
[Clipboard](#)
[Details](#)

Display Show Sort by Send to

About Entrez

Text Version

All: 1 Review: 0

Entrez PubMed

Overview

Help | FAQ

Tutorial

New/Noteworthy

E-Utilities

PubMed Services

Journals Database

MeSH Database

Single Citation Matcher

Batch Citation Matcher

Clinical Queries

Special Queries

LinkOut

My NCBI (Cubby)

Related Resources

Order Documents

NLM Catalog

NLM Gateway

TOXNET

Consumer Health

Clinical Alerts

ClinicalTrials.gov

PubMed Central

☐ 1: Virology. 1988 Oct;166(2):379-86.

[Related Articles, Links](#)

Stress-induced increase of hexose transport as a novel index of cytopathic effects in virus-infected cells: role of the L protein in the action of vesicular stomatitis virus.

Pasternak CA, Whitaker-Dowling PA, Widnell CC.

Department of Biochemistry, St. George's Hospital Medical School, London, United Kingdom.

The VSV-specific increase in hexose transport by BHK cells has been measured by assay of the $[3H]dGlc/[14C]AIB$ uptake ratio. The effect was abolished by uv-irradiation of the virus, indicating that viral gene expression is required. Cells infected with the T1026 R1 mutant of VSV, which causes only slight cytopathic changes, exhibited only a slight increase in hexose uptake. Cells infected with temperature-sensitive (ts) mutants of VSV that are defective in the function of the viral N, NS, G, or M proteins at the restrictive temperature (39.5 degrees) exhibited increased $[3H]dGlc/[14C]AIB$ uptake ratios typical of wild-type virus at either restrictive (39.5 degrees) or permissive temperature (34 degrees). Cells infected with a mutant defective in the function of the viral L protein exhibited an increased $[3H]dGlc/[14C]AIB$ uptake ratio at permissive temperature (34 degrees) only; at restrictive temperature (39.5 degrees) the uptake ratio was essentially the same as that of mock-infected cells. Temperature-shift experiments indicated that the effect on hexose transport persisted for at least 6 hr in cells which no longer expressed function L protein, and that when expression of L was restricted to the first 2 hr of infection, an almost complete stimulation of hexose transport was observed 4 hr later. These results indicate that expression of the L gene is a necessary factor for inducing an increased hexose uptake in VSV-infected BHK cells. They also suggest that the action of the L protein on hexose transport is indirect, and is presumably mediated by other cellular constituents. The studies support the concept that an increased dGlc uptake may be a useful index of the cytopathic consequences of virus infection.

PMID: 2845651 [PubMed - indexed for MEDLINE]

Phase I Trial of Intravenous Administration of PV701, an Oncolytic Virus, in Patients With Advanced Solid Cancers

By Andrew L. Pecora, Naiyer Rizvi, Gary I. Cohen, Neal J. Meropol, Daniel Sterman, John L. Marshall, Stuart Goldberg, Peter Gross, James D. O'Neil, William S. Groene, M. Scot Roberts, Harvey Rabin, Michael K. Bamat, and Robert M. Lorence

Purpose: PV701, a replication-competent strain of Newcastle disease virus, causes regression of tumor xenografts after intravenous administration. This phase I study was designed to define the maximum-tolerated dose (MTD) and safety of single and multiple intravenous doses of PV701 as a single agent in patients with cancer.

Patients and Methods: Seventy-nine patients with advanced solid cancers that were unresponsive to standard therapy were enrolled. Four PV701 intravenous dosing regimens were evaluated: (1) single dose: one dose every 28 days; (2) repeat dose: three doses in 1 week every 28 days; (3) desensitizing: one lower dose followed by two higher doses in 1 week every 28 days; and (4) two week: one lower dose followed by five higher doses over 2 weeks every 21 days.

Results: A 100-fold dose intensification was achieved over 195 cycles. A first-dose MTD of 12×10^9 plaque-forming units (PFU)/m² was established for out-

patient dosing. After an initial dose of 12×10^9 PFU/m², patients tolerated an MTD for subsequent doses of 120×10^9 PFU/m². The most common adverse events were flu-like symptoms that occurred principally after the first dose and were decreased in number and severity with each subsequent dose. Tumor site-specific adverse events and acute dosing reactions were also observed but not cumulative toxicity. Objective responses occurred at higher dose levels, and progression-free survival ranged from 4 to 31 months. Tumor tissue from one patient was obtained after 11 months of therapy and showed evidence of PV701 particles budding from the tumor cell membrane by electron microscopy and a pronounced lymphoplasmacytic infiltrate by histologic examination.

Conclusion: PV701 warrants further study as a novel therapeutic agent for cancer patients.

J Clin Oncol 20:2251-2266. © 2002 by American Society of Clinical Oncology.

PV701^{1,2} IS A HIGHLY purified, replication-competent, naturally attenuated strain of Newcastle disease virus,³⁻⁶ an avian paramyxovirus. Newcastle disease virus strains, such as PV701, directly lyse diverse human cancer cells in vitro (oncolytic) while being significantly less toxic toward normal human cells.^{1,3,7} Moreover, the virus is capable of both stimulating T-cell-mediated specific antitumor immunity and inducing nonspecific activation of immune function, such as the induction of cytokines (eg, interferon) and activation of tumoricidal macrophages.⁸⁻¹⁰

Newcastle disease virus is a rapidly replicating RNA virus with progeny virions first detectable in vitro within 3 hours after infection. After infecting a cancer cell, the virus rapidly spreads to neighboring tumor cells through the release of progeny virions and syncytia formation.^{3,11} PV701 and certain other negative strand RNA viruses are selectively cytolytic for tumor cells as a result of defects in the interferon (IFN) signaling pathway that are common among diverse tumor types.^{12,13} Defects in this pathway are believed to confer a growth and survival advantage to tumor cells.¹³⁻¹⁶ However, these tumor defects also disable the antiviral function of IFN and confer sensitivity of malignant cells to infection and replication of viruses such as PV701.

Oncolytic Newcastle disease virus strains, including PV701, administered via intravenous, intraperitoneal, and intratumoral routes, replicate selectively in human cancer cells implanted in athymic mice, resulting in high rates of

complete tumor regression and sparing of normal tissue.^{2,3,17,18} In vitro death for most tumor cell lines occurs at a PV701 amount ~1,000-fold below the amount that adversely affects normal cells.¹ Similarly, there is an ~1,000-fold difference in the intravenous dose resulting in 50% tumor regression in athymic mice ($\sim 2 \times 10^6$ plaque-forming units [PFU]/mouse) and the median lethal dose ($\sim 5 \times 10^9$) in these mice.² In these animals, clear dose and dose frequency effects are observed, providing the rationale for examining such effects in the clinical setting. Objective

From the Cancer Center at Hackensack University Medical Center, Hackensack, NJ; Lombardi Cancer Center, Georgetown University Medical Center, Washington, DC; The Cancer Center at Greater Baltimore Medical Center, Baltimore, and Pro-Virus, Inc., Gaithersburg, MD; and Fox Chase Cancer Center and University of Pennsylvania Medical Center, Philadelphia, PA.

Submitted August 6, 2001; accepted February 4, 2002.

Supported by Pro-Virus, Inc., Gaithersburg, MD, through Clinical Trial Agreements.

Presented in part at the Thirty-Seventh Annual Meeting of the American Society of Clinical Oncology, San Francisco, CA, May 12-15, 2001.

Address reprint requests to Andrew L. Pecora, MD, Hackensack University Medical Center, Northern New Jersey Cancer Center, 20 Prospect Ave, Suite 400, Hackensack, NJ 07601; email: apecora@humed.com.

© 2002 by American Society of Clinical Oncology.

0732-183X/02/2009-2251/\$20.00

responses (complete response and partial response) increase from ~50% to 100% by raising the dose three-fold or increasing the number of doses given at the same dose level from one to three doses.⁷ In addition, intravenous exposure to a lower first dose of PV701 (3×10^8 PFU/mouse) results in a desensitization of the animal to toxicity from subsequent doses as evidenced by a 10-fold increase in the maximum-tolerated dose (MTD) for dose 2 (10^{10} PFU/mouse) compared with dose 1 (10^9 PFU/mouse). The oncolytic effect of PV701 requires live replication-competent virus because UV-inactivated virus caused no tumor regression.²

Oncolytic activity associated with Newcastle disease virus was first observed by Cassel and Garrett¹⁹ when an intratumoral injection in a patient with cervical cancer resulted in tumor regression of the injected mass as well as a supraclavicular lymph node metastasis. Subsequent clinical trials of Newcastle disease virus focused primarily on a vaccine approach using viral oncolysates that included low doses of viable virus infecting autologous tumor cells.^{8,20,21}

Characteristics of Newcastle disease virus that are favorable for human trials include the genetic stability of vaccine strains, the absence of genetic recombination, lack of a carrier state of naturally attenuated strains, and the lack of antigenic drift.³ Human-to-human transmission has not been observed.⁶ The virus has been safely given to humans in tumor vaccine studies, and accidental exposure has been reported to cause only self-limiting conjunctivitis.^{3,5,6}

Other replication-competent viruses, including adenovirus and herpes simplex virus alone or in combination with chemotherapy, have caused tumor regressions in humans by the intratumoral route.²²⁻²⁴ Early testing of both replication-competent and replication-incompetent adenoviruses by the intravenous route has been initiated,^{25,26} but, until now, there has been no determination of the MTD for systemic therapy with a virus. Herein we report on a phase I study with dose escalations including the testing of various treatment schedules and MTD determination for systemic (intravenous) administration of the replication-competent virus PV701 in patients with advanced cancer that was unresponsive to standard therapy.

PATIENTS AND METHODS

Patient Enrollment

Seventy-nine patients were enrolled with advanced or metastatic solid malignancy that was unresponsive to treatment with established therapies. Entry criteria included at least one bidimensionally measurable tumor, ≥ 18 years of age, a life expectancy of at least 3 months, and a performance status (Eastern Cooperative Oncology Group) of 0 or 1. Laboratory result minimum entry requirements included 3,000 WBCs/ μ L, 1,500 neutrophils/ μ L, and 100,000 platelets/ μ L. Also

required was a serum creatinine level less than 1.5 times the upper limit of normal, serum transaminase level less than 2.5 times the upper limit of normal (< 5.0 times the upper limit of normal for patients with metastatic liver disease), and a therapy-free period of 14 days. Patients were not eligible if documented to have CNS disease (including brain tumors on computed tomography [CT]/magnetic resonance imaging [MRI] scans required at screening), known hypersensitivity to eggs, antiviral or systemic corticosteroid treatment within 14 days, myocardial infarction or life-threatening arrhythmia within 6 months, known positivity for human immunodeficiency virus, active hepatitis B or C infection, an organ allograft, autoimmune disease, active viral infection (including cold or influenza), or uncontrolled bacterial infection. Active poultry workers and pregnant or nursing women were excluded. All patients provided informed written consent approved by the appropriate institutional review board.

PV701

PV701 is a highly purified isolate of the naturally attenuated (nonrecombinant) MK107 vaccine strain of Newcastle disease virus and is distinct from other strains, such as 73-T, that have been previously tested in humans. PV701 was cloned from MK107 by biologic means (triple-plaque purification in chicken embryo cells) to increase homogeneity and to remove defective particles. PV701 was grown to high titer in specific pathogen-free embryonated chicken eggs (SPAFAS, Inc, Preston, CT) and purified from allantoic fluid. Clinical lots of PV701 used in this study met release criteria, including potency, purity, sterility, and adventitious agent testing (Investigational New Drug Application BB 7401, May 7, 1998).

Plaque Assay

Doses were expressed as the amount of infectious virus in PFU. For the plaque assay, HT1080 human fibrosarcoma cells (obtained from American Type Culture Collection, Manassas, VA) were seeded into six-well tissue culture plates and grown to confluence. The growth medium was removed, monolayers were washed with serum-free medium, and 0.5 mL of various sample dilutions were added per well. The plates were incubated with rocking for 90 minutes at 37°C and 5% CO₂. The medium was completely removed, the monolayers were washed as above, and 3 mL of semisolid agar medium was overlaid onto each well. The cultures were incubated for 48 hours at 37°C and 5% CO₂. Monolayers were stained with neutral red for counting of plaques, and the virus titer was expressed as PFU/mL.

Intravenous Administration of PV701

For the first 47 patients, PV701 was prepared in a sterile syringe and patients were administered the dose over 10 minutes (regardless of dose level) by injection of the syringe contents into a port on a running intravenous line (at ~25 mL/h). For the subsequent 32 patients, PV701 was diluted into an intravenous saline bag (50 to 100 mL) immediately before dosing and administered at a rate of 1.2×10^9 PFU/m²/min for doses of 12×10^9 PFU/m² and at a rate of 5.0×10^9 PFU/m²/min for doses greater than 12×10^9 PFU/m².

Treatment and Study Design

Single-dose regimen. A single dose of 5.9, 12, or 24×10^9 PFU/m² was administered every 28 days ($n = 17$ patients). The starting dose of 5.9×10^9 PFU/m² was selected because this dose was greater than 1 log below the rodent MTD on the basis of body surface area. Dose

escalation proceeded by two-fold increments. All patients were hospitalized for 24 hours with intensive monitoring.

Repeat-dose regimen. Two dose levels were examined ($n = 13$ patients): either 5.9×10^9 PFU/m² or 12×10^9 PFU/m² was administered three times in 1 week every 28 days. Dose 2 was given 2 days after dose 1.

Desensitizing regimen. Five dose levels were examined ($n = 37$ patients): all patients received 12×10^9 PFU/m² (desensitizing dose) on the first day of administration followed by two doses of 24×10^9 PFU/m², two doses of 48×10^9 PFU/m², two doses of 72×10^9 PFU/m², two doses of 96×10^9 PFU/m², or two doses of 144×10^9 PFU/m². Dose 2 was given 2 days after dose 1. For each patient, all three doses were administered within 1 week and repeated every 28 days.

Two-week regimen. Two dose levels were examined ($n = 12$ patients): all patients received 12×10^9 PFU/m² (desensitizing dose) on the first day of administration followed by five doses of 96×10^9 PFU/m² or five doses of 120×10^9 PFU/m². Dose 2 was given 4 days after dose 1. Patients were given three doses per week for 2 weeks (six total doses) followed by 1 week off treatment. Enrollment was for a minimum of two courses.

General. Patients were monitored before each treatment and extensively after treatment. Evaluations included physical examinations, measurement of performance status, laboratory parameters, viral shedding (urine and sputum), and serum testing for PV701 antibodies and infectious virus. CT or MRI was used to assess tumor responses after each course of therapy.

A minimum of three patients were entered at each PV701 dose level until a patient experienced a dose-limiting toxicity (DLT). When a DLT was encountered, three additional patients were enrolled at that same dose level. There was no further dose escalation when two or more patients experienced a DLT. The MTD was defined as the dose level below that at which two or more DLTs were encountered.

Adverse events were graded using the Southwest Oncology Grading Scale. DLT was defined as a clinically significant adverse event (grade 4 leukocyte or neutrophil count lasting > 5 days; platelet count $< 10,000/\mu\text{L}$ [grade 4 by NCI common toxicity criteria version 2.0]; or \geq grade 3 nonhematologic excluding fever and fatigue). Transient increases in hepatic transaminases ($> \text{grade 2}$) without grade 2 hyperbilirubinemia were not considered a DLT if these elevations returned to baseline before the next course. Symptoms clearly related to disease progression were not considered as DLTs.

All patients were eligible for additional courses of treatment when they had at least stable disease and an acceptable toxicity profile.

Virologic Studies

Samples of urine and sputum were screened for infectious virus by examining for cytopathogenic effects in cultures of HT1080 cells. For these screening assays of urine and sputum, spiked samples with as little as 100 PFU/mL and 100 PFU/g, respectively, were positive. All positive samples were quantified by plaque assay on HT1080 cells (as described in Plaque Assay section).

Neutralizing Antibody to PV701

Two-fold dilutions of heat-inactivated patient serum were mixed with a PV701 preparation that contained 3×10^2 PFU/mL in a total volume of 2 mL. After incubation for 1 hour at room temperature, 0.5 mL of the serum-virus mixture was tested for infectivity using the plaque assay described above. The neutralizing antibody titer of the

serum sample is expressed as the last dilution resulting in at least 80% reduction in the number of plaques.

Cytokine Measurements

Patient serum was analyzed using commercially available enzyme-linked immunosorbent assay kits for detection of human cytokines (IFN- α and IFN- γ , BioSource International, Camarillo, CA; IFN- β , Fujirebio, Inc, Tokyo, Japan; interleukin-6 [IL-6] and tumor necrosis factor- α [TNF- α], Pierce-Endogen, Rockford, IL). Sera from 10 patients were analyzed including two patients who were given a single dose of 12×10^9 PFU/m², one patient who was given a single dose of 24×10^9 PFU/m², five patients who were given three repeat doses of 12×10^9 PFU/m², and two patients in the desensitizing regimen who were given a first dose of 12×10^9 PFU/m² followed by two doses of 24×10^9 PFU/m².

Tissue Processing

Sections of formalin-fixed patient tissues were processed for hematoxylin and eosin (H&E) staining. Electron microscopy of patient tissue samples was performed by negative staining with uranyl acetate and compared with those from experimental HT1080 human fibrosarcoma xenografts infected with PV701.²

Statistical Analysis

Assessments of the association between age or baseline anemia and grade 3 flu-like symptoms, the association of transaminase elevations (> 200 U/L) and preexisting liver metastases, and the association of preexisting lung disease and oxygen desaturation were performed using Fisher's exact test. The null hypothesis was that the probability of an adverse event was the same in patients with and without the baseline characteristic. A two-sided alternative was considered statistically significant at $P < .05$.

RESULTS

Patient Characteristics

Seventy-nine patients (48 men and 31 women) with advanced cancer that was unresponsive to standard therapy were enrolled onto this study (Tables 1 and 2) from June 1998 through September 1999 and were treated over 12 dose levels with a total of 195 cycles. The median age was 58 years (range, 24 to 81 years) with 22 patients (28%) older than 70 years. The most common primary tumor types were colorectal ($n = 23$), pancreatic ($n = 9$), renal ($n = 9$), breast ($n = 8$), and non-small-cell lung carcinoma ($n = 8$). Seventy-two patients had received previous chemotherapy; 35 of them received three or more regimens.

Antibody Response

Thirty-two patients were tested at baseline for neutralizing antibody to PV701. One of these patients was positive at the limit of detection of the assay. His adverse event profile was no different from that of other study subjects. The other 31 patients tested all were negative for neutralizing antibody.

Table 1. Dosage Levels

Dose Level	Regimen	Doses ($\times 10^9$ PFU/m ²)	Dose Intensity ($\times 10^9$ PFU/m ² /course)	No. of Patients Enrolled	No. of Cycles
1	Single	5.9	5.9	6	19
2	Single	12	12	6	11
3	Single	24	24	5	13
4	Repeat	5.9 \times 3	17.7	6	8
5	Repeat	12 \times 3	36	7	11
6	Desensitizing	12, 24 \times 2	60	4	10
7	Desensitizing	12, 48 \times 2	108	3	37*
8	Desensitizing	12, 72 \times 2	156	5	14
9	Desensitizing	12, 96 \times 2	204	12	28
10	Desensitizing	12, 144 \times 2	300	13	13
11	Two-week	12, 96 \times 5	492	7	18
12	Two-week	12, 120 \times 5	612	5	13
Total				79	195

*This cohort includes patient no. 521, who received 34 cycles of PV701 including his most recent 25 cycles at 12/120 \times 5 (10^9 PFU/m²).

Fourteen of 16 patients in the single-dose regimen, seven of seven patients in the repeat-dose regimen, and six of six patients in the desensitizing-dose regimen became seropositive, first evident 1 to 2 weeks after PV701 dosing. By week 4 after initial dosing, 10 of 12 patients tested in the repeat-dose and desensitizing regimens had neutralizing antibody titers at 1:320 to 1:640. Eight patients were tested at 5 to 10 weeks after initial dosing, and they had a median neutralizing antibody titer of 1:640 (range, 1:80 to 1:2,560). One patient was followed over 18 courses (1.5 years). At month 3, his titer reached a plateau (at 1:2,560) that has persisted through the last time point analyzed (month 18).

Virology

A total of 821 sputum samples and 899 urine samples from 67 patients were examined for virus shedding using highly sensitive infectivity assays. Positive samples were quantified by plaque assay. Fewer than 1% of the sputum samples tested were positive. These positive samples contained low PV701 levels (median 26 PFU/g sputum). No PV701 was detectable in the sputum at day 14 or beyond. All repeat course sputum samples were negative. Fifteen percent of all urine samples tested positive at a low level (median, 820 PFU/mL), and all samples were negative 3 weeks after the last dose. Five percent of urine samples analyzed from courses 2 to 6 were positive for PV701. The percentage of patients with transient viremia at any time during a course dropped from a first course high of 54% to 0% in patients who received seven or more courses of PV701.

Cytokines

Serum samples from 10 representative patients who were given one or multiple doses (12 or 24 $\times 10^9$ PFU/m²) were

analyzed at multiple time points for serum proinflammatory cytokines (IFN- α , IFN- β , IFN- γ , IL-6, and TNF- α). Similar patterns of cytokine production were noted for all 10 patients after their first dose of PV701. IFN- α was the predominant cytokine produced in all patients (median peak levels, 20,000 pg/mL) compared with the four other cytokines (median peak levels between 10 and 200 pg/mL). Detectable increases in IFN- α , IFN- γ , IL-6, and TNF- α were first seen by 6 hours after dosing with IFN- β only detectable at 20 hours after dosing. All four cytokines consistently reached peak levels at 20 hours after dosing and returned to or near baseline by 2 to 3 days after dosing. In patients who received more than one dose of PV701, there was a marked reduction in serum cytokine levels after the second dose compared with the levels seen after the first dose (eg, for IFN- α , a median peak of 13,000 pg/mL noted after the first dose compared with a median peak of 65 pg/mL after the second dose).

Toxicity

Most common adverse events. Throughout the trial, fever, other cytokine-related flu-like symptoms (eg, chills, fatigue, nausea/vomiting, headache, diarrhea), and hypotension were the most common adverse events (Tables 3 and 4), primarily occurring 4 to 24 hours after PV701 dosing. Except for the immediate dosing reactions (detailed below), adverse events diminished markedly in number and severity with repeat dosing (Table 4) and with the second (see Table 3) and all subsequent courses.

Two of the first three patients in the first cohort (5.9 $\times 10^9$ PFU/m²) had grade 3 fever of 40.0°C to 40.6°C, which was promptly reversed with ibuprofen. Beginning with the fifth patient in this cohort, all subsequent patients in this trial received acetaminophen and ibuprofen prophylaxis and the

Table 2. Patient Characteristics

	No. of Patients
Total No.	79
Age, years	
Median	59
Range	24-81
Male/female	48/31
Primary tumor site	
Colorectal	23
Pancreatic	9
Renal	9
Breast	8
Non-small-cell lung	8
Sarcoma	4
Head and neck	4
Melanoma	3
Mesothelioma	2
Esophageal	2
Lymphoma	2
Ovarian	1
Bladder	1
Carcinoma (unknown primary)	1
Cholangiocarcinoma	1
Carcinoid	1
No. of prior chemotherapy regimens	
0	7
1	13
2	24
≥ 3	35
No. of prior immunotherapy regimens	
0	60
1	9
2	10
No. of prior hormonal therapy regimens	
0	69
1	2
≥ 2	8
No. of prior investigational agents	
0	61
1	11
≥ 2	7
Prior surgery	68
Prior radiation therapy	40

incidence of grade 3 fever was reduced to 11% (eight of 75 patients).

In the single-dose regimen, 42% of patients (seven of 17) had at least one episode of diarrhea including one case of grade 4. In subsequent dosing regimens, diarrhea was effectively controlled using loperamide with 10% of patients having diarrhea.

Age and baseline anemia were examined in all 79 patients as potential risk factors for grade 3 flu-like symptoms (fever, fatigue, nausea, vomiting, and dehydration). Age was examined because two DLTs at the $12/144/144 \times 10^9$ billion PFU/m² dose level occurred in elderly patients (81

and 75 years of age; see Dose Escalations, DLT, and Determination of MTD below). Anemia was examined as a risk factor because it might exacerbate the severity of any fatigue, the most common of the grade 3 flu-like symptoms. The analysis showed that 13 (59%) of 22 patients ≥ 70 years of age had grade 3 flu-like symptoms compared with 18 (32%) of 57 less than 70 years of age ($P < .025$), and 24 (52%) of 46 patients with baseline anemia (Hgb < 11 or Hct < 35) had grade 3 flu-like symptoms compared with seven (21%) of 33 patients without baseline anemia ($P < .01$).

Desensitization to toxicity on repeat dosing. As predicted from the animal models, dose 1 desensitized patients to the flu-like symptoms on subsequent doses. Table 4 lists the six most common adverse events observed for patients in the desensitizing regimen in order of decreasing incidence and by the Southwest Oncology Group severity grade for each dose. Adverse events were reduced in number and severity after the second and third doses despite a two-fold to eight-fold increase in dose. With all patients receiving prophylactic antipyretics, the incidence of grade 3 fever for patients in this regimen was reduced from 13% on dose 1 to being undetected with subsequent doses (Table 4). The incidence of grade 1 to 2 fever reduced from 83% with dose 1 to 17% with dose 3. A similar pattern of desensitization to adverse events was seen in the 2-week dosing regimen with doses 2 to 6 producing milder and less frequent adverse events compared with dose 1, even when doses 2 to 6 were eight- to 10-fold higher (data not shown). This desensitization to toxicity with repeat doses was also seen for the hematologic changes (see Hematology/Coagulation Profiles below).

Acute dosing reactions. Acute and reversible dosing reactions were observed in five of the first seven patients enrolled at the $12/96/96 \times 10^9$ PFU/m² dose level, typically during the third dose of the first course. These reactions consisted of back pain, chest tightness, chest pain, and hypertension. Abdominal pain was less commonly seen. In all cases, the onset was within 5 minutes of the start of dosing and resolved spontaneously and completely within 30 minutes of the beginning of the adverse event. In a few instances, these adverse events required a pause in the administration of virus. One patient experienced grade 3 back pain on his third PV701 injection and was the only patient in the study who did not complete a PV701 dosing because of this acute dosing reaction. All other acute dosing reactions were grade 1 or 2. These reactions were attributed to the rate of administration of the virus, which had increased from 1.2×10^9 PFU/m²/min at the 12×10^9 PFU/m² dose level to 1.0×10^{10} PFU/m²/min at the higher dose levels. In subsequent patients, the administration rate for doses above 12×10^9 PFU/m² was decreased to 5×10^9

Table 3. Adverse Events for All Patients During the First Two PV701 Courses

Adverse Event	Course 1 (N = 79)			Course 2 (n = 39)		
	Grade 1/2 (%)	Grade 3 (%)	Grade 4 (%)	Grade 1/2 (%)	Grade 3 (%)	Grade 4
Flu-like symptoms						
Fever	92	13	—	59	3	—
Chills	73	—	—	44	—	—
Fatigue/malaise	70	32	—	26	3	—
Headache	34	1	—	5	—	—
Myalgia	23	—	—	13	—	—
Diaphoresis	19	—	—	13	—	—
Gastrointestinal						
Nausea	72	1	1	26	—	—
Vomiting	57	4	1	23	—	—
Anorexia	54	—	—	18	—	—
Diarrhea	53	1	3	15	—	—
Constipation	16	1	—	5	—	—
Dehydration	15	9	—	—	—	—
Dry Mouth	10	—	—	—	—	—
Hematologic						
Thrombocytopenia	46	13	3*	—	—	—
Anemia	38	8	1	13	3	—
Leukopenia	37	23	4†	18	—	—
Increased PT/PTT	30	1	1	15	—	—
Neutropenia	23	8	1	8	—	—
Cardiovascular						
Hypotension	52	5	1	15	—	—
Edema, peripheral	15	3	—	13	—	—
Dizziness	14	—	—	—	—	—
Respiratory						
Dyspnea/hypoxia	27	8	6	15	3	—
Cough	18	—	—	5	—	—
Liver						
Increased ALT/AST	47	18	4	18	3	—
Increased bilirubin	4	10	3	—	—	—
Neurologic						
Pain, back/flank	24	8‡	—	15	3	—
Pain, abdominal	24	4	—	18	3	—
Confusion/disorientation	18	3	—	—	—	—
Anxiety/agitation	13	—	—	5	—	—
Pain, hip/leg	10	—	—	13	—	—
Acute dosing reaction						
Back pain	19	1	—	26	—	—
Chest pain	11	1	—	18	—	—
Metabolic						
Hypokalemia	10	—	—	5	—	—

NOTE. Included are all events with at least a 10% incidence for any grade.

*For both cases of SWOG grade 4 thrombocytopenia (platelet nadir at 23,000/ μ L in a patient dosed with a single dose of 24×10^6 PFU/ m^2 and platelet nadir at 19,000/ μ L in a patient dosed at 12/96/ 96×10^6 PFU/ m^2), platelets had recovered to a grade 1 to 2 level within 4 to 6 days and no bleeding was observed.

†All three cases of grade 4 leukopenia occurred 20 hours after dosing and had recovered to grade 2 at the next time point (3 days later).

‡Grade 3 pain occurred in patients with baseline pain at this location.

PFU/ m^2 /min (see Patients and Methods). At the slower administration rate, dosing reactions occurred infrequently in subsequent patients and were less severe. The symptoms of back pain, chest tightness, and hypertension were suggestive of a vasospasm effect, although no ECG changes were observed and prophylaxis with antihistamines was found to be ineffective.

Tumor site-specific adverse events including inflammation. A separate class of adverse events dependent on the tumor location was noted. These tumor site-specific adverse events included the following:

- Tumor inflammation/edema on physical examination (one patient with a scalp metastasis from a colon carcinoma, the other with tongue carcinoma).

Table 4. Percentage of the Six Most Common Adverse Events Observed for Patients Given Three PV701 Doses for Which Doses 2 and 3 Were Up to Eight-Fold Higher Than Dose 1 (n = 24 patients, desensitizing regimen)

Adverse Event	Dose 1 (n = 24)		Dose 2 (n = 23)		Dose 3 (n = 23)		Dose 1, Course 2 (n = 14)	
	Grade 1-2 (%)	Grade 3 (%)	Grade 1-2 (%)	Grade 3 (%)	Grade 1-2 (%)	Grade 3 (%)	Grade 1-2 (%)	Grade 3 (%)
Fever	83	13	82	0	17	0	27	0
Chills	54	0	39	0	17	0	20	0
Nausea	46	8	22	0	30	0	13	0
Fatigue	41	38	21	4	26	9	7	0
Vomiting	38	8*	4	0	17	0	7	0
Hypotension	33	0	26	0	4	0	0	0

NOTE: Includes all patients at dose levels 12/24/24 (four patients), 12/48/48 (three patients), 12/72/72 (five patients), and 12/96/96 $\times 10^9$ PFU/m² (12 patients).

*In addition, there was one case of grade 4 vomiting.

- Oxygen desaturation observed only in patients with lung/pleural tumor involvement (13 of 55 patients with pulmonary tumor involvement v zero of 24 without involvement; $P < .01$).
- Pulmonary adverse events (grade ≥ 3 , including six cases of grade 3 dyspnea) in nine of 55 patients with pulmonary tumor involvement. Seven of these nine patients had one or more of the following baseline characteristics (signs, symptoms, radiographic evidence): baseline grade 2 dyspnea, lung tumors at least 5 cm in size, significant pleural effusions, partial or complete lobectomy, lobar atelectasis, and lobar consolidation. There were no clinically significant pulmonary adverse events observed in the 24 patients without lung involvement by tumor.
- Transiently elevated liver transaminases over 200 U/L occurred only in patients with liver metastases (18 of 38 patients with liver metastases v zero of 41 patients without; $P < .01$).
- An enterocutaneous fistula at the tumor site (with the tumor extending from bowel to skin surface) in a 63-year-old man with a colon carcinoma occurred 9 days after his first dose of 144×10^9 PFU/m².

Hematology/coagulation profiles. After the first dose of the first course of PV701, all patients experienced a transient drop in leukocyte and platelet counts with full recovery to baseline observed within 7 to 14 days, regardless of dose level. Clinically significant thrombocytopenia (Southwest Oncology Group grade 4, nadirs of 19,000 and 23,000 platelets/ μ L) and leukopenia (grade 4) were observed in two and three patients, respectively. However, these patients were carefully monitored clinically with follow-up hematology profiles performed 12 hours to 4 days later and a rapid recovery in blood counts was observed in all cases. No episodes of bleeding or infection resulted from these transient drops in counts. The pattern of thrombocytopenia

and leukopenia was identical for patients who were given one dose as for those who were given up to six doses, indicating that this phenomenon was due to the first PV701 dose. The rate of recovery was the same for the single-dose patients as for those who were given multiple PV701 doses with full recovery noted by day 14 in all patients, including those who were given subsequent doses up to 10 times higher than dose 1. There were no significant changes in leukocyte and platelet counts during subsequent courses.

There were no significant changes in hemoglobin or hematocrit values after PV701 dosing in patients without baseline anemia. Grade 3 anemia was reported for six patients, all of whom had significant anemia at baseline.

Specific assays were added to the standard coagulation panel to serve as early predictors of potential disseminated intravascular coagulation (ie, fibrinogen and fibrin split products). There were no dose- or time-related changes in these parameters or in the standard coagulation parameters (prothrombin time, partial thromboplastin time) that were considered clinically significant and related to therapy.

Hypoglycemia in patients on oral hypoglycemic agents or insulin. Three instances of clinically significant hypoglycemia occurred. All three patients had diabetes (two were receiving oral hypoglycemic agents, and one was receiving insulin). After the initial PV701 dose, these patients experienced nausea and dehydration, resulting in limited oral intake. Hypoglycemia was not observed after subsequent PV701 dosing. It is unknown whether PV701 administration also increased the bioavailability of the hypoglycemic therapy. Discontinuing these agents in the immediate post-dosing period after dose 1 resulted in no additional episodes of hypoglycemia.

Dose escalations, DLT, and determination of MTD. In the single-dose regimen, doses between cohorts were escalated in two-fold increments from 5.9 to 24×10^9 PFU/m². As can be seen in Table 5, one adverse event (grade 4

Table 5. Dose-Limiting Toxicities

Dose Level	Regimen	Doses ($\times 10^9$ PFU/m ²)	Dose Being Escalated	No. of Patients Enrolled	No. of Patients With DLT	Type of DLT
1	Single	5.9	First dose	6	1	1 pt with diarrhea (gr 4)
2	Single	12	First dose	6	0	None
3	Single	24	First dose	5	1*	1 pt with dyspnea† (gr 3) and hypoglycemia (gr 3)
4	Repeat	5.9×3	N/A‡	6	1	1 pt with dyspnea§ (gr 4)
5	Repeat	12×3	N/A‡	7	0	None
6	Desensitizing	$12, 24 \times 2$	Second dose	4	0	None
7	Desensitizing	$12, 48 \times 2$	Second dose	3	0	None
8	Desensitizing	$12, 72 \times 2$	Second dose	5	0	None
9	Desensitizing	$12, 96 \times 2$	Second dose	12	1	1 pt with acute dosing reaction (gr 3 back pain)
10	Desensitizing	$12, 144 \times 2$	Second dose	13	3¶	1 pt with tremors (gr 3) and dehydration (gr 3); 1 pt with dehydration (gr 3); 1 pt with hypoxia (gr 3) which occurred during rigors
11	Two-week	$12, 96 \times 5$	N/A‡	7	0	None
12	Two-week	$12, 120 \times 5$	N/A‡	5	0	None#

Abbreviations: DLT, dose-limiting toxicity; pt, patient; gr, grade; N/A, not applicable.

*Grade 2 hypotension was also observed at this dose level (in three of five patients) and was the only dose-dependent toxicity in the single-dose regimen. Dose escalation was stopped to allow an outpatient dosing regimen. The dose of 12×10^9 PFU/m² was therefore established as the outpatient MTD for the first dose with grade 2 hypotension as dose limiting.

†This patient with baseline compromised lung function had worsening of underlying pulmonary infiltrate after PV701 dosing. See the Results, which describes an association of lung/pleural tumor involvement and respiratory adverse effects.

‡Tolerance of repeat dosing tested rather than dose escalation.

§This patient with baseline extensive lung metastases and bilateral pleural effusions experienced grade 4 dyspnea after the first dose of 5.9×10^9 PFU/m² and did not experience any recurrence with doses 2 and 3 or during a second course.

¶One patient of the first seven of this cohort had an acute dosing reaction (grade 3 back pain) due to the 96×10^9 PFU/m² dose. The infusion rate was slowed and five more patients enrolled onto this dose level and only one mild dosing reaction occurred (grade 1 abdominal discomfort).

#Dose escalation of the second dose was stopped at 144×10^9 PFU/m² due to the occurrence of three dose-limiting toxicities in this cohort.

||One patient with preexisting compromised lung function died of respiratory failure after receiving only the desensitizing dose of 12×10^9 PFU/m². There were no dose-limiting toxicities seen with the dose of 120×10^9 PFU/m².

diarrhea) that met the definition of DLT was seen in the first cohort (5.9×10^9 PFU/m²) of six patients. Severe diarrhea was not seen on subsequent higher dose levels when loperamide was given prophylactically at the first sign of gastrointestinal side effects. No DLTs were seen in six patients in the 12×10^9 PFU/m² cohort. At the 24×10^9 PFU/m² dose level, a DLT (grade 3 dyspnea) occurred in a patient with a lung tumor mass and baseline signs of pulmonary infiltrate. This patient also experienced grade 3 hypoglycemia. Dyspnea and hypoglycemia were not considered dose dependent in this trial because severe dyspnea was associated with patients having lung/pleural tumor masses and hypoglycemia only occurred in patients with diabetes (discussed in Hypoglycemia in Patients on Oral Hypoglycemic Agents or Insulin).

In the single-dose regimen, the only dose-dependent toxicity was grade 2 hypotension, which occurred in three of five patients in the 24×10^9 PFU/m² cohort. Because the intention was to establish an outpatient dosing regimen, dose 1 was not escalated further. The dose of 12×10^9

PFU/m² was therefore established as the outpatient MTD for the first dose with grade 2 hypotension as dose limiting.

The repeat-dose regimen tested for the presence of any cumulative toxicity at two dose levels ($5.9/5.9/5.9$ and $12/12/12 \times 10^9$ PFU/m²) in a total of 13 patients. As indicated in Table 5, only a single DLT was observed (grade 3 dyspnea), which occurred in a breast cancer patient with baseline bilateral pleural effusions dosed at $5.9/5.9/5.9 \times 10^9$ PFU/m², the lower of the two dose levels. No cumulative toxicity was seen. A dose of 12×10^9 PFU/m² was therefore chosen as a first dose (or "desensitizing dose") for escalation of the second and subsequent doses in the desensitizing regimen.

In the desensitizing regimen, one DLT was observed in the first four cohorts with a total of 24 patients (Table 5). This acute dosing reaction (grade 3 back pain) at $12/96/96$ was attributed to the infusion rate (see Acute Dosing Reactions above), which was slowed for subsequent patients. In the $12/144/144 \times 10^9$ PFU/m² cohort of 13 patients, three DLTs were observed and dose escalation was

stopped. These events were seen after the 144×10^9 PFU/m² dose: grade 3 tremors and dehydration in an 81-year-old woman, grade 3 dehydration in a 75-year-old man, and grade 3 hypoxia associated with 30 minutes of rigors in a man with lung cancer.

For patients in the 2-week regimen, repeat doses lower than 144×10^9 PFU/m² were therefore tested. There was no significant difference in adverse event profile or laboratory values for repeat doses of 96 or 120×10^9 PFU/m² given five times over 2 weeks, no cumulative toxicity was seen, and patients tolerated equally well either of these doses. Therefore, the dose of 120×10^9 PFU/m² was determined to be the second dose MTD.

Serious Adverse Events and Deaths

Seven cases of dehydration requiring intravenous fluids and/or hospitalization were noted. Most of these cases were associated with significant nausea/vomiting and/or diarrhea. These events were reversible and did not result in lasting effects. As discussed above, patients over age 70 were at increased risk for flu-like grade 3 adverse events.

Three patients with baseline bacterial infections were administered PV701 and had episodes of sepsis after PV701 therapy. Two patients had a baseline urinary tract infection. The other patient had baseline fever in the week before beginning PV701 treatment and had baseline bacteremia immediately before his first dose of PV701.

There were five patient deaths, four of which were clearly attributed to progressive disease occurring during the 4-week reporting period. The remaining death occurred in a 55-year-old man with renal carcinoma metastatic to the lungs. At baseline, he had compromised pulmonary function as a result of previous lobectomies, lobar atelectasis, and an 8-cm metastasis in one of the two remaining lobes. In addition, he was status postradical nephrectomy with a 4-cm tumor metastasis in his remaining adrenal gland. This patient was enrolled in the $12/120 \times 5$ doses $\times 10^9$ PFU/m² dose level. After an initial (and only) PV701 dose of 12×10^9 PFU/m², he experienced grade 3 hypotension that required intravenous hydration and 48 hours of hospitalization. Three days later, he was admitted to a local community hospital with complaints of fatigue, lethargy, and severe respiratory distress. Mechanical ventilation was advised but was declined. The patient died as a result of respiratory failure approximately 12 hours later. At autopsy, an enlarged subcarinal lymph node ($5 \times 4 \times 3$ cm) filled with partially hemorrhagic and necrotic tumor tissue was reported as well as a metastatic tumor that measured $8 \times 7 \times 6$ cm just below the inferior pleural surface of the left upper pulmonary lobe. Histologic sections of the left lung were reported as showing the presence of localized thrombi only

in the tumor vessels with tumor necrosis and severe edema/inflammation only in the tumor-bearing lung and mild to absent in the non-tumor-bearing lung. Inflammation was not reported in any other organ.

Response Assessment

Seventeen patients were not eligible for response assessment. Twelve patients were removed from the study before a radiographic response assessment because of toxicity or worsening baseline symptoms. Three patients were taken off the study for progressive disease. One patient was removed as a result of noncompliance, and one was removed to receive radiation therapy.

Of the 62 patients who were eligible for response assessment, 14 had freedom from tumor progression for 4 to 30+ months and two had radiographic evidence of major responses. A complete response was documented in a 51-year-old man with tonsillar (squamous cell) carcinoma. At the time of enrollment, this patient had disease progression during cisplatin and radiation therapy (with the most recent treatment given in September 1998) as noted by a radiographic increase during the preceding 3 months. After a baseline MRI scan in January 1999 (Fig 1A) demonstrating a 1.5-cm tumor in the pharynx, he received PV701 at the $12/96/96 \times 10^9$ PFU/m² dose level. After one cycle, he achieved a radiographic complete response as evidenced by resolution of the tumor on MRI. Follow-up scans after months 2, 3, and 5 of therapy confirmed the radiographic complete response (Fig 1B). The patient was noncompliant and discontinued therapy between months 5 and 7. An MRI scan at month 7 indicated disease progression elsewhere in the pharynx (lateral oropharyngeal wall).

A partial response was documented in a 79-year-old man who had colon carcinoma and had failed capecitabine, 5-FU, and irinotecan. He had not received any chemotherapy in the 2 months before his enrollment into the PV701 study at the $12/72/72 \times 10^9$ PFU/m² dose level. At baseline, he had two liver metastases, the largest one well-circumscribed and measuring 10 cm in maximal dimension (Fig 2A). His CT scans at month 1 (Fig 2B) and month 2 after therapy showed overall tumor regression of greater than 70%. In addition, immediately after dosing there was a spike in carcinoembryonic antigen level (approximately 4.5-fold increase) followed by a drop to steady-state levels at 79% below baseline, shown elsewhere to be further indicative of a response.²⁷ Progression-free survival of 10 months was observed in this patient. Seven other patients with diverse malignancies (including mesothelioma, melanoma, colon carcinoma, breast carcinoma, pancreatic carcinoma, and carcinoma) had measurable tumor reduction, although less than 50% of the total tumor burden.



Fig 1. Complete response in a man with tonsillar carcinoma. (A) Baseline MRI image showing a 1.5-cm tumor at the junction of the right tongue base and tonsillar pillar. (B) MRI image at month 3 showing complete resolution of tumor.

Replacement of Tumor by Inflammatory Cells on Histologic Examination

During the course of PV701 therapy, one patient had tumor tissue removed for electron microscopic examination and other tissue analysis. This 46-year-old man, who had bulky peritoneal mesothelioma that had progressed after debulking surgery and intraperitoneal doxorubicin/cisplatin and IFN- γ , was enrolled at the $12/48/48 \times 10^9$ PFU/m 2 dose level in January 1999. During 30 monthly courses of PV701, he has remained free from progression, has no disease-related symptoms, experienced an improvement in performance status (to Eastern Cooperative Oncology Group 0), gained muscle mass, and retained a high level of physical activity. CT scans performed on a monthly basis

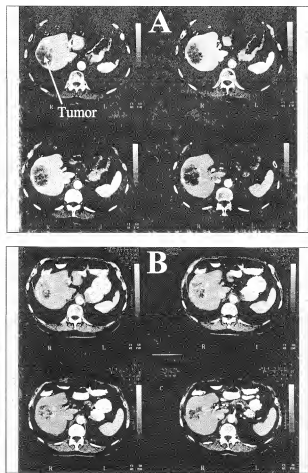


Fig 2. Partial response in a man with colon carcinoma. (A) Baseline CT scan demonstrating 10-cm liver metastasis. (B) CT scan at month 1 demonstrating the response.

have shown up to a 35% reduction in bidimensional measurable disease (270 cm 2 at study entry). Elective surgery to debulk disease 2 weeks after his last dose of the eighth course (in the eleventh month of PV701 administration) was unsuccessful. However, histologic examination showed a significant fraction of the tumor mass replaced by an active inflammatory process with edema in all sections of tumor (Fig 3A and 3B). This process consisted predominantly of plasma cells. Lymphoid follicles with germinal centers were also evident in the tumor, indicating an active immune reaction (Fig 3C). Electron microscopy revealed PV701 particles at tumor cell membranes (Fig 3D). The plasma cell infiltrate and secondary lymphoid follicles were not present in previous sections of tumor parenchyma taken before enrollment. A normal skin sample removed at the time of the patient's tumor excision did not show any

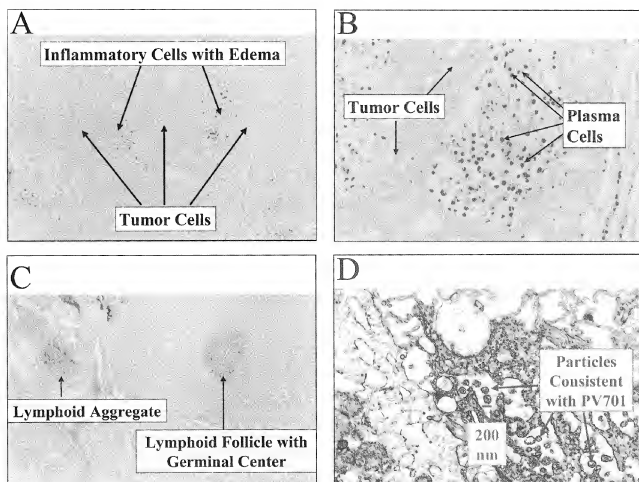


Fig 3. Microscopic tumor sections after 8 PV701 courses. Tumor parenchyma (H&E histologic stain) showing (A) inflammation/edema, (B) plasma cell infiltrate, and (C) lymphoid follicle/aggregate. (D) Electron micrograph shows particles consistent with PV701 budding from the tumor cell membrane.

evidence of inflammation. His serum neutralizing antibody titer had reached a plateau level of 1:2,560 at the time of the tissue examination.

In another case, a different pattern of tumor inflammation was seen. Autopsy sections from a patient who had advanced pleural mesothelioma and died of progressive disease (tumor obstruction of the inferior vena cava) were reviewed. Lung metastases displayed a mononuclear inflammatory infiltrate mainly at the periphery of the larger metastases and throughout the smaller tumor masses (Fig 4A and 4B). Also observed in the lung metastases were signs of tumor necrosis, including multifocal areas at the tumor periphery (Fig 4A). The portion of lung without tumor was free of any signs of inflammation (Fig 4C). A similar pattern was seen in his liver metastasis (Fig 4D), which showed mononuclear inflammatory cells infiltrating

the tumor but not uninvolved liver distal from the tumor (Fig 4E). A diffuse mononuclear inflammatory infiltrate was also seen in a mesenteric metastasis (Fig 4F) but not in the adjacent normal tissue. No such inflammatory process was present in the original biopsy of the primary tumor preceding PV701 treatment. Unlike the previous case of the patient with peritoneal mesothelioma, there was no sign of tumor regression in this particular patient and no samples of tumor tissue were obtained for viral analysis.

DISCUSSION

Among the various clinical tests of replication-competent viruses^{22-25,28,29} (Stojdl et al, manuscript submitted for publication), the phase I dose escalation study reported here is the first study in humans to determine an MTD for systemic (intravenous) administration. PV701, an oncolytic

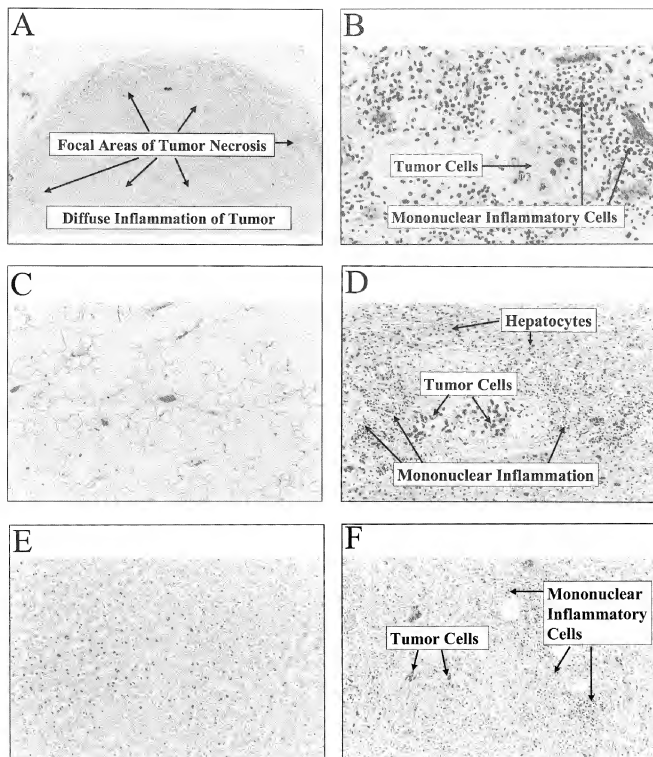


Fig 4. Histologic (H&E) tissue sections from a patient with pleural mesothelioma. [A, B] Lung metastasis showing inflammation and multifocal necrosis. [C] Lung uninvolved by tumor. [D] Liver metastasis showing inflammation. [E] Liver uninvolved by tumor. [F] Mesenteric metastasis showing inflammation.

strain of Newcastle disease virus was studied because it demonstrated preclinical activity against a wide range of human tumors *in vitro* and *in vivo* and is active by the intravenous route. Importantly, Newcastle disease virus has previously been shown to lack pathogenicity in humans after low-dose administration^{3,8,20,21,30} mainly as a component of oncolysate tumor vaccines.^{8,20,21} This phase I study characterized the toxicity profile for intravenous PV701 dosing and demonstrated the feasibility and potential benefit of systemic (intravenous) oncolytic virus administration.

As expected from previously published reports on Newcastle disease virus serologic surveys,^{31,32} only one of the 32 patients tested was found to have neutralizing antibody before administration of the virus. After dosing with PV701, the majority (27 of 29) of patients tested developed varying levels of neutralizing antibody. Neutralizing antibody titers, even with multiple courses, reached a moderate plateau level of ~1:2,560, including a patient who received repeated cycles of PV701 for more than 18 months. Of potential clinical significance, signs of efficacy (eg, tumor regressions) were observed in patients after formation of these antibody titers.

Low levels of viral shedding were observed and found generally to be transient. Recovery of virus from sputum was rare, was of low level, occurred only after the first cycle of virus administration, and always cleared within a maximum of 14 days. Recovery of virus from urine after the first cycle of PV701 was more common but again did not persist, being cleared within 3 weeks. Transient viremia was observed less frequently after subsequent cycles but did occur despite the presence of neutralizing antibodies. Ultimately, the incidence of transient viremia diminished to zero among patients who received PV701 for seven or more cycles. Relative to the environmental impact of shedding on the most susceptible host species (chickens), the observed levels of PV701 shedding are orders of magnitude below the standard avian vaccine dose required for an antibody response.^{33,34}

The low and transient viral shedding seen in this study may be part of the explanation for the lack of any observed human-to-human transmission seen with PV701. This finding is in agreement with data from other clinical trials using Newcastle disease virus³ and with other human experience with the virus.^{3,6}

The acute toxicity of PV701 principally consisted of flu-like symptoms (fever, chills, fatigue, headache, nausea, vomiting, and diarrhea) and dose-dependent hypotension that occurred 4 to 24 hours after PV701 dosing. Older patients (≥ 70 years) and those with anemia (hemoglobin < 11 g/dL) were found to be more likely to experience flu-like symptoms of greater severity. Acute toxic effects have been

shown in animal models to be a result of Newcastle disease virus-induced release of proinflammatory cytokines, including type I IFNs and TNF- α .³⁵⁻³⁹ Levels of type I IFN, IFN- γ , TNF- α , and IL-6 were elevated in patients in this study after the first dose of PV701, with levels first detected by 6 hours and peaking at hour 20. This pattern paralleled the time course of the flu-like symptoms (eg, fever was consistently noted between hours 4 and 20). Antipyretics and anti-diarrheal agents reduced the incidence and severity of these toxicities.

Just as tachyphylaxis develops in mammals, including humans, with repeat IFN and TNF dosing,⁴⁰⁻⁴² a striking reduction in the incidence and severity of PV701-mediated acute toxicity on repeat dosing was observed (Table 4). This phenomenon, termed "desensitization," was first observed with PV701 in the rodent models and applies to effects on toxicity but not efficacy. In preclinical testing using human tumor xenografts in athymic mice, efficacy increased with repeat dosing and toxicity was markedly reduced. After intravenous administration of 3×10^8 PFU, mice tolerated subsequent intravenous doses of 1×10^{10} PFU, indicating at least a 10-fold increase in the MTD.³ As fully predicted by these preclinical studies, this desensitization phenomenon allowed a 10-fold increase in the MTD observed in this trial with a first dose MTD of 12×10^9 PFU/m² and a second dose MTD of 120×10^9 PFU/m². The reduction in adverse event profile seen with the second PV701 dose compared with the first dose paralleled the reduction in serum cytokine levels seen after second PV701 dose, suggesting a causative role of proinflammatory cytokines in the clinical toxicity of PV701. As preclinically in both immunodeficient and immunocompetent mice, this desensitization phenomenon in patients does not depend on the development of antibodies to PV701 because it is seen as early as 2 days after the first PV701 dose (when antibodies are not detectable).

Desensitization also occurred with respect to transient drops in platelet and WBC counts. IFN and TNF- α are known to cause transient changes in blood cell counts as a result of margination.^{40,43-47} A previous study by Merrigan et al,³⁰ using single doses (from 10^6 to 10^8 PFU/patient) of Newcastle disease virus, orders of magnitude below the doses tested in this trial, verified a dose-dependent induction of IFN in the serum of 17 patients along with fever and a transient leukopenia. In the present study, these transient hematologic changes were induced by the first dose of PV701. Leukocyte and platelet levels recovered during the first course of repeat dosing, even when doses were 10-fold higher than the first dose. Importantly, the leukopenias and thrombocytopenias were not correlated with signs of infection or bleeding, and the degree and rate of recovery did not

require therapeutic intervention. The lack of cumulative effects seems to be consistent with margination of leukocytes and platelets rather than a consumptive process.

There was no observed cumulative toxicity associated with prolonged repeated PV701 dosing including a total of 116 repeat courses given to a total of 39 patients. One patient has had more than 30 courses of PV701 with no evidence of an adverse effect on any organ system. Because of desensitization and the lack of cumulative toxicity, an overall dose intensification of more than 100-fold was achieved in this trial (Table 1).

Tumor site-specific inflammatory reactions were also seen. In this study, two patients with palpable tumors (colon cancer with a scalp metastasis and tongue cancer) developed inflammation and edema localized to the tumor sites. Histologic confirmation of tumor site-specific inflammation was obtained from representative patients. In one patient with metastatic pleural mesothelioma, tumor necrosis and a mononuclear cell inflammatory infiltrate were observed only at tumor sites without any involvement of normal tissue, including tissue adjacent to disease sites (Fig 4). Evidence of such tumor inflammation as seen in this trial raises a question about the determination of responses by traditional imaging criteria, especially in future phase II trials.

Tumor site-specific effects were also observed in patients with tumor involvement of the lung and liver. Oxygen desaturation, for example, was observed only in patients with pulmonary/pleural-based tumors (13 of 55 with involvement *v* zero of 24 without; $P < .01$). Significant elevation in liver enzymes was also limited, occurring only in patients with liver metastases (18 of 38 *v* zero of 41; $P < .01$). In addition, there was no evidence of generalized hepatocyte toxicity because total serum protein and clotting times remained comparable to baseline. Furthermore, the typical pattern of liver enzymes (elevated gamma glutamyl-transferase and alkaline phosphatase disproportionate to transaminases; and $AST > ALT$) was indicative of pressure on the canaliculi and cholangioles from a space-occupying effect rather than hepatocellular damage from hepatitis.^{48,49} Of cautionary note for future trials, patients with malignancy extensively replacing normal lung tissue, particularly if baseline pulmonary dysfunction exists, seem to be at risk for severe pulmonary toxicity. One such patient with pre-existing compromised lung function died of respiratory failure. Severe edema and inflammation was found local-

ized to the tumor-bearing lung along with thrombosis confined to the tumor vessels.

Response assessment was not the focus of this phase I study, especially because, in this dose escalation study, most patients received low, potentially suboptimal dose intensities (Table 1). However, 62 patients were assessable. Evidence of efficacy included progression-free survival from 4 to more than 30 months in 14 patients who had clear evidence of disease progression before initiation of PV701. Furthermore, two radiographic objective responses (complete response and partial response) were documented and seven other patients had measurable tumor reductions, although not to the degree of a partial response. A 46-year-old man with advanced peritoneal mesothelioma unresponsive to intraperitoneal chemotherapy, with bulky disease (four 8- to 10-cm masses with total bidimensional measurable disease of 270 cm²) at baseline, has received more than 30 courses of PV701, has maintained an improved performance status (Eastern Cooperative Oncology Group 0), has had a radiographic minor response (of 35% tumor regression), and has experienced no cumulative toxicity. Evidence of a direct oncolytic effect of PV701 in this patient was found on biopsy after 11 months of PV701 administration. PV701 particles were observed budding from tumor cell membranes, and the tumor mass was extensively filled with mononuclear inflammatory cells (especially plasma cells) replacing tumor, indicating that PV701 had gained access to the tumor and was replicating there despite the presence of serum neutralizing antibody. In comparison to this patient with one of the largest tumor burdens enrolled onto the study, the patient with the smallest tumor burden (1.5 cm, tonsillar cancer) experienced a complete radiographic response after three doses of PV701.

Collectively, these observations support the concept that systemic therapy with the replication-competent virus PV701 can provide a novel and potentially important therapy for patients with solid tumors, including those unresponsive to standard therapy. Moreover, long-term intravenous virus therapy seems to be feasible in humans and may play an important role in the treatment of solid tumors. Additional clinical studies of PV701 have begun.

ACKNOWLEDGMENT

We thank the dedicated nurses for patient care and study coordination. Phoebe L. Stewart, PhD, UCLA School of Medicine, is gratefully acknowledged for independent review of the electron micrographs. We thank John Bell, PhD, for permission to cite his unpublished review article on oncolytic viruses.

REFERENCES

- Roberts S, Buasen PT, Incao BA, et al: PV701, a naturally attenuated strain of Newcastle disease virus, has a broad spectrum of oncolytic activity against human tumor xenografts. *Proc Am Assoc Cancer Res* 42:454, 2001 (abstr 2441)
- Lorence RM, Roberts S, Groene WS, et al: Regression of human tumor xenografts following intravenous treatment using PV701, a naturally attenuated oncolytic strain of Newcastle disease virus. *Proc Am Assoc Cancer Res* 42:454, 2001 (abstr 2442)
- Lorence RM, Roberts MS, Groene WS, et al: Replication-competent, oncolytic Newcastle disease virus for cancer therapy, in Hernaiz Driever P, Rabkin SD (eds): *Replication-Competent Viruses for Cancer Therapy*. Monographs in Virology (vol. 22). Basel, Switzerland, Karger, 2001, pp 160-182
- Nelson NJ: Scientific interest in Newcastle disease virus is reviving. *J Natl Cancer Inst* 91:1708-1710, 1999
- Kleiman MB: Newcastle disease virus, in Lennette EH (ed): *Laboratory Diagnosis of Viral Infections*. New York, NY, Marcel Dekker, 1992, pp 567-571
- Alexander DJ: Newcastle disease and other avian paramyxoviruses. *Rev Sci Tech* 9:443-462, 2000
- Reichard KW, Lorence RM, Cascino CJ, et al: Newcastle disease virus selectively kills human cancer cells. *J Surg Res* 52:448-453, 1992
- Schirmacher V, Ahlert T, Probstle T, et al: Immunization with virus-modified tumor cells. *Semin Oncol* 25:677-696, 1998
- Termeer CC, Schirmacher V, Brocher EB, et al: Newcastle disease virus infection induces B7-1/B7-2-independent T-cell costimulatory activity in human melanoma cells. *Cancer Gene Ther* 7:316-323, 2000
- Schirmacher V, Bai L, Umansky V, et al: Newcastle disease virus activates macrophages for anti-tumor activity. *Int J Oncol* 16:363-373, 2000
- Reichard KW, Lorence RM, Cascino CJ, et al: Selective replication of Newcastle disease virus in cancer cells is associated with virus-induced cell fusion. *Proc Am Assoc Cancer Res* 33:521, 1992 (abstr)
- Roberts MS, Lorence RM, Groene WS, et al: Treatment of neoplasms with viruses. International Patent Publication No. WO 99/18799, 1999
- Stojdi DF, Licht B, Knowles S, et al: Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus. *Nat Med* 6:821-825, 2000
- Jaramillo ML, Abraham N, Bell JC: The interferon system: A review with emphasis on the role of PKR in growth control. *Cancer Invest* 13:327-338, 1995
- Stark GR, Kerr IM, Williams BR, et al: How cells respond to interferons. *Ann Rev Biochem* 67:227-264, 1998
- Gil J, Esteban M: Induction of apoptosis by the dsRNA-dependent protein kinase (PKR): Mechanism of action. *Apoptosis* 5:107-114, 2000
- Lorence RM, Reichard KW, Katubig BB, et al: Complete regression of human neuroblastoma xenografts in athymic mice after local Newcastle disease virus therapy. *J Natl Cancer Inst* 86:1228-1233, 1994
- Lorence RM, Katubig BB, Reichard KW, et al: Complete regression of human fibrosarcoma xenografts after local Newcastle disease virus therapy. *Cancer Res* 54:6017-6021, 1994
- Cassel WA, Garrett RE: Newcastle disease virus as an antineoplastic agent. *Cancer* 18:863-868, 1965
- Cassel WA, Murray DR: Treatment of stage II malignant melanoma patients with Newcastle disease virus oncolysate. *Nat Immun Cell Growth Regul* 7:351-352, 1998
- Batiwalla FM, Bateman B, Serrano D, et al: A 15-year follow up of AJCC stage III malignant melanoma patients treated post-surgically with Newcastle disease virus (NDV) oncolysate and determination of alterations in the CD8 T cell repertoire. *Mol Med* 4:783-794, 1998
- Khuri FR, Nemunaitis J, Ganly I, et al: A controlled trial of intratumoral ONYX-015, a selectively-replicating adenovirus, in combination with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer. *Nat Med* 6:879-885, 2000
- Markert JM, Medlock MD, Rabkin SD, et al: Conditionally replicating herpes simplex virus mutant, G207, for the treatment of malignant glioma: Results of a phase I trial. *Gene Ther* 7:867-874, 2000
- Ganly I, Kim D, Eckhardt SG, et al: A phase I study of Onyx-015, an E1B attenuated adenovirus, administered intratumorally to patients with recurrent head and neck cancer. *Clin Cancer Res* 6:798-806, 2000
- Nemunaitis J, Cunningham C, Buchanan A, et al: Intravenous infusion of a replication-selective adenovirus (ONYX-015) in cancer patients: Safety, feasibility and biological activity. *Gene Ther* 8:746-759, 2001
- Hao D, Rowinsky EK, Smetzer LA, et al: A phase I and pharmacokinetic study of intravenous (IV) p53 gene therapy with RPR/INGN-201 in patients (pts) with advanced cancer. *Proc Am Soc Clin Oncol* 20:262a, 2001 (abstr 1045)
- Quayle JB: Tumour lysis as a factor affecting blood levels of CEA. *Br J Cancer* 46:213-219, 1982
- Kim D: Systemic administration of replication-selective adenoviruses. *Eur J Cancer* 37:S65, 2001 (suppl 3, abstr 105)
- Cattaneo R (ed): Replicating vectors for gene therapy. *Gene Ther* 8:S1-S13, 2001 (suppl 1)
- Merigan TC, DeClercq E, Finkelstein MS, et al: Clinical studies employing interferon inducers in man and animals. *Ann N Y Acad Sci* 173:746-759, 1970
- Miller LT, Yates VJ: Reactions of human sera to avian adenoviruses and Newcastle disease virus. *Avian Dis* 15:781-788, 1971
- Charan S, Mahajan VM, Agarwal LP: Newcastle disease virus antibodies in human sera. *Indian J Med Res* 73:303-307, 1981
- Newcastle disease vaccine. United States Code of Federal Regulations. Title 9, Part 113, Section 329, 1999
- Thorton DH: Quality control of vaccines, in Alexander DJ (ed): *Newcastle Disease*. Boston, MA, Kluwer Academic Publishers, 1988, pp 347-365
- Raj NB, Cheung SC, Rosztochy I, et al: Mouse genotype affects inducible expression of cytokine genes. *J Immunol* 148:1934-1940, 1992
- Baron S, Buckler CE: Circulating interferon in mice after intravenous injection of virus. *Science* 141:1061-1063, 1963
- Brehm G, Kirchner H: Analysis of the interferons induced in mice in vivo and in macrophages in vitro by Newcastle disease virus and by polyinosinic-polycytidylic acid. *J Interferon Res* 6:21-28, 1986
- Busam KJ, Bauer TM, Bauer J, et al: Interleukin-6 release by rat liver macrophages. *J Hepatol* 11:367-373, 1990
- Lorence RM, Rood PA, Kelley KW: Newcastle disease virus as an antineoplastic agent: Induction of tumor necrosis factor- α and augmentation of its cytotoxicity. *J Natl Cancer Inst* 80:1305-1312, 1988

40. Savage PD, Muss HB: Renal cell cancer, in DeVita VT, Hellman S, Rosenberg SA (eds): *Biologic Therapy of Cancer* (ed 2). Philadelphia, PA, JB Lippincott, 1995, pp 373-387
41. Fraker DL, Stovroff MC, Merino MJ, et al: Tolerance to tumor necrosis factor in rats and the relationship to endotoxin tolerance and toxicity. *J Exp Med* 168:95-105, 1988
42. Fraker DL, Pass HI, Alexander HR, et al: Biologic therapy with TNF: Systemic administration and isolation-perfusion, in DeVita VT, Hellman S, Rosenberg SA (eds): *Biologic Therapy of Cancer* (ed 2). Philadelphia, PA, JB Lippincott, 1995, pp 329-345
43. Chapman PB, Lester TJ, Casper ES, et al: Clinical pharmacology of recombinant human tumor necrosis factor in patients with advanced cancer. *J Clin Oncol* 5:1942-1951, 1987
44. Creagan ET, Ahmann DL, Green SJ, et al: Phase II study of recombinant leukocyte A interferon (rIFN- α A) in disseminated malignant melanoma. *Cancer* 54:2844-2849, 1984
45. Creaven PJ, Brenner DE, Cowens JW, et al: A phase I clinical trial of recombinant human tumor necrosis factor given daily for five days. *Cancer Chemother Pharmacol* 23:186-191, 1989
46. Feldman ER, Creagan ET, Schaid DJ, et al: Phase II trial of recombinant tumor necrosis factor in disseminated malignant melanoma. *Am J Clin Oncol* 15:256-259, 1992
47. Quesada JR, Talpaz M, Rios A, et al: Clinical toxicity of interferons in cancer patients: A review. *J Clin Oncol* 4:234-243, 1986
48. Pincus MR, Schaffner JA: Assessment of liver function, in Henry JB (ed): *Clinical Diagnosis and Management by Laboratory Methods* (ed 19). Philadelphia, PA, WB Saunders, 1996, pp 253-267
49. Pincus MR, Zimmerman HJ, Henry JB: Clinical enzymology, in Henry JB (ed): *Clinical Diagnosis and Management by Laboratory Methods* (ed 19). Philadelphia, PA, WB Saunders, 1996, pp 268-295

Integrating pharmacology and *in vivo* cancer models in preclinical and clinical drug development

J.K. Peterson, P.J. Houghton*

Department of Molecular Pharmacology, St. Jude Children's Research Hospital, Memphis, TN, USA

Received 23 October 2003; received in revised form 19 December 2003; accepted 8 January 2004

Abstract

Historically, cancer drug development has been a roller coaster. Numerous agents have shown exciting activity in preclinical models and yet have had minimal activity clinically. These disappointments have led to reasonable scepticism about the true value of both syngeneic and xenograft rodent tumour models in accurately identifying agents that will have important clinical utility. Whereas the development of newer techniques, including transgenic mouse models of cancer, offers the potential to develop more predictive models, the role of such mice in cancer drug development is not yet validated. To advance in our understanding of predictive model systems it may be wise to analyse both the successes and the failures of conventional models in order to understand some of their limitations and perhaps to avoid making the same mistakes in the future. Here we review the value and limitations of xenograft models, and the role of integrating preclinical pharmacology in developing new treatments for solid tumours of childhood.

© 2004 Elsevier Ltd. All rights reserved.

Keywords: Paediatric cancer; Pharmacokinetics; Pharmacodynamics; Molecular targets; Xenografts

1. Introduction

The evaluation of antitumour agents in immune-deficient mice (athymic nude or severe combined immunodeficient (*scid*) mice) transplanted with human tumours is the major model system for drug development. In its most simple iteration, tumours are grown subcutaneously, and the model allows rapid and quantifiable assessment of antitumour activity relative to mouse toxicity. Logically, precedence should be given to those agents that show the greatest antitumour activity in the preclinical setting, assuming the preclinical data are predictive of drug activity in human studies. The challenge lies in being able to extrapolate these results to the clinic. Indeed, can this ever be done with any degree of confidence? The extensive screening for over 10 years by the National Cancer Institute (NCI) suggests only a moderate predictive value for their xenograft models, and even less concordance between *in vitro* testing data and clinical utility [1]. In this analysis, xenograft tumours derived from a particular cancer type did not

predict for activity in the respective clinical disease; rather broad-spectrum activity in the preclinical models was associated with greater clinical activity. Interestingly, these results recapitulate those from syngeneic rodent tumour models used in the NCI screening programme before 1985, where clinical activity was associated with a high response rate in five of eight unrelated solid tumour models. The deficiency in all of these studies has been an inability or failure to relate tumour-response data to clinically achievable drug systemic exposures (i.e. studies on pharmacokinetics were not undertaken).

There are many reasons why preclinical results do not predict human efficacy. Here we will focus on differences in interspecies pharmacology. However, it is clear that the design of therapeutic clinical trials often fails to build upon the strong preclinical leads that may guide aspects of clinical trials' design, such as scheduling of drug administration. Conversely, criteria used to advance an agent in preclinical trials may not be as stringent as those used to evaluate response rates in the clinical setting. For example, 58% inhibition of tumour growth, a criterion used by NCI for assessing the activity of a drug against xenograft tumour models, represents progressive disease in a clinical trial. Our data suggest that if certain aspects of the study design are

* Corresponding author. Tel.: +1-901-495-3440; fax: +1-901-521-1668.

E-mail address: peter.houghton@stjude.org (P.J. Houghton).

given careful consideration, it may be valid to predict clinical results derived from preclinical work with the xenograft model. These aspects include (a) the development of early-passage models of the appropriate human cancer, rather than the use of 'ancient' cell lines that have been in culture for decades; (b) the use of clinically relevant response criteria to evaluate a new entity; (c) the assessment of tumour responsiveness relative to drug systemic exposure; and (d) a rational consideration of the major/minor strengths and weaknesses of the model (i.e. all models have certain limitations). Here we review our experience using models of childhood cancers in drug development.

2. Retrospective studies: validation of tumour models

Xenograft tumour models, in which a human cancer is transplanted into immune-deficient mice, have been explored since the mid 1960s, but became more frequently used after the identification of the athymic nude mutant mouse which is deficient in T cells [2,3]. The more recent discovery of other immune-deficient mouse strains has further expanded the options for host transplantation. For example, the non-obese diabetic (NOD) *scid* mouse has proved useful for the propagation and testing of agents against acute lymphocytic leukaemias established from children [4]. It has been well recognised that when human cancers are transplanted into mice they retain many characteristics of the original tumour (histology, chromosomal abnormalities, surface antigen expression). Although subcutaneous tumours metastasise infrequently this rate is increased when transplanted to orthotopic (natural) sites. However, from the perspective of drug sensitivity, at least to the conventional cytotoxic agents that comprise most of our current experience, the subcutaneous models appear relatively predictive. That is, agents known to be active in a clinical

ical disease can be identified as active in the models. Examples are shown in Table 1, and summarise the preclinical and clinical activities of vincristine, cyclophosphamide, actinomycin D and doxorubicin against a panel of childhood rhabdomyosarcomas [5,6], and also colon adenocarcinomas (unpublished data). When one uses objective response (i.e. partial response or $\geq 50\%$ volume regression) as the criterion for 'activity' the results are rather interesting. The rhabdomyosarcoma 'model' identifies agents known to be active in this disease, whereas the same agents have little activity against colon carcinoma xenografts. This, at least, indicates that tumour responsiveness is not merely a consequence of heterografting tumour into the mouse. We have now established comprehensive panels of different childhood solid tumours and where there are adequate data to make reasonable judgments, similar correlations have been found. For example, atypical teratoid rhabdoid tumours (ATRT) of the central nervous system or kidney are essentially resistant to all agents tested in the model, consistent with their known clinical sensitivity. In contrast, xenografts of histologically favourable Wilms' tumours (nephroblastoma) established from patients before treatment are exquisitely sensitive to vincristine, actinomycin D and cyclophosphamide (J. Dome, P. Houghton, data not shown) agents that are used in the curative treatment of this cancer. Wilms' tumours established as xenografts from patients at relapse are far less sensitive to these agents, consistent with previous observations with models of rhabdomyosarcoma and acute lymphocytic leukaemia [4,5]. Poor-prognosis Wilms' tumours having diffuse anaplastic histology are also poorly responsive as xenografts to conventional chemotherapy agents. Thus, there seems to be a reasonable correlation between drug activity in these preclinical models and their known activity against the same clinical disease. So, if there is such a good *retro*-predictive correlation, what goes wrong when we try to extrapolate from preclinical to clinical activity?

3. Prospective use of xenograft models

The first prospective use of xenograft data followed perhaps our observation that melphalan had very significant activity against the 'diagnosis' panel of rhabdomyosarcomas [7]. In a phase II clinical trial in 13 patients who had failed standard chemotherapy, melphalan demonstrated marginal activity (one partial response). However, pharmacokinetic analysis showed that exposure to this agent in children was essentially similar to that in mice at doses inducing tumour regression. Consequently, we extended the trial to include children at diagnosis with very advanced disease and a dismal long-term outcome [8]. Against disease at diagnosis melphalan demonstrated very significant activity

Table 1
Sensitivity of rhabdomyosarcoma and colon adenocarcinoma xenografts to conventional cytotoxic agents

Agent/tumour type	Objective response rate (%) in the model	Objective response rate (%) in the clinic
<i>Rhabdomyosarcoma*</i>		
Vincristine	78	59
Cyclophosphamide	44	54
Actinomycin D	11	24
Doxorubicin	19	31
<i>Colon carcinoma</i>		
Vincristine	0	< 10
Cyclophosphamide	0	< 10
Actinomycin D	0	< 10
Doxorubicin	0	< 10
5-fluorouracil	17	15-20
Methyl CCNU	17	15-20

* References [5,6] (and references therein).

(10 objective responses in 13 patients). This study was important for two reasons. It taught us that the pre-clinical model used to select an agent for clinical trial should closely mimic that patient population against which it will be tested (i.e. diagnosis models may not predict for relapse), and that comparative drug exposures may be one metric that should be considered in predicting clinical antitumour activity. Some examples of drugs that showed activity in preclinical childhood tumours, and their success or failure in clinical trials, are summarised in Table 2. DMP840, Carzelesin, and Sulophenur each showed activity in the preclinical model [9–11], but failed to demonstrate significant activity in either phase I or II clinical trials. Examination of the systemic exposure (area under the concentration-time curve; AUC) to each agent at the maximum tolerated dose (MTD) in mice and that achieved at the MTD in phase I testing is shown in the table, as is the range of drug doses (relative to the MTD in mice) over which the agents caused objective tumour regressions. For these agents it would be predicted that adequate systemic exposure in patients would not be attained to achieve significant antitumour activity. For example, even at the minimum dose of DMP840 causing tumour regressions in the mouse (approximately 30% of the MTD) the systemic exposure is still 5-fold higher in rodents than can be achieved in patients. In contrast, it would be anticipated that exposures to melphalan, topotecan and irinotecan (in this case the active metabolite SN-38) would be adequate to anticipate clinical antitumour activity. Indeed, as with melphalan, both topotecan and irinotecan are highly active drugs in the treatment of rhabdomyosarcoma [12–14]. This approach has been extended to the design of clinical trials [15] in which a retrospective analysis of the exposure to topotecan required to induce objective regressions in most neuroblastoma xenografts [16] was used to define an optimal target for drug exposure in children. Preliminary results indicate that the doses in children can be adjusted to achieve the exposures that caused regressions in this model of relapsed neuroblastoma [15]. Further, clinical responses in this trial are consistent with the predictive value of the model. In a subsequent phase II clinical trial, targeting the daily

topotecan lactone exposure to achieve 100 ng/h per ml yielded a high response rate (58%) in newly diagnosed patients with advanced neuroblastoma, consistent with that predicted by the neuroblastoma xenograft models [17].

The evaluation of the illudin S derivative Irofulven (MGI-114) serves to illustrate how pharmacokinetic studies may be valuable in making informed decisions on clinical development. As shown in Table 3, Irofulven shows rather dramatic activity against a panel of 20 tumour xenografts derived from various types of brain tumour, neuroblastoma and rhabdomyosarcoma [18]. Indeed at the MTD (approximately 4.6 mg/kg), objective regressions in 14 or 18 models were obtained. However, at 1.3 mg/kg this agent demonstrated poor activity, causing regression in only one of 14 tumour models examined. At dosages in mice ranging from 1.3 to 7.0 mg/kg (4.0–22 mg/m²), the daily systemic exposure to Irofulven ranged from 214 to 1152 ng/h per ml. This is compared with the systemic exposure associated with the maximally tolerated dosage from the adult phase I clinical trial of Eckhardt and colleagues [19] (Fig. 1). For the clinical trial the mean (\pm SD) daily AUC for Irofulven at the MTD were approximately

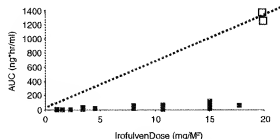


Fig. 1. Relation between Irofulven systemic exposure (area under the curve; AUC) in man and mouse. Data, replotted from Eckhardt and colleagues [18], show the daily AUC in individual patients enrolled in a phase I clinical trial of Irofulven (closed symbols). Shown also are AUC determined in mice following administration of Irofulven (21 mg/m²) to mice not bearing tumours (open symbols). Data are from Leggas *et al.* [17]. The dashed line shows the approximate AUC in the mouse over a dose range similar to that in patients (assuming linear pharmacokinetics). At the minimum effective dose in mice (4 mg/m²) the systemic exposure still significantly exceeds that in patients at the maximum tolerated dose (MTD) (15 mg/m²).

Table 2
Retrospective analysis of drug response-exposure correlations in childhood tumour models

	Agent	AUC at mouse MTD AUC at human MTD	Effective dose range from the mouse MTD ^a
'Clinical failures'	DMP840	15–20	approximately 2–3
	Carzelesin	Approx. 80	<2
	Sulophenur	Approx. 8	Approx. 3
'Clinically active'	Melphalan	1	Approx. 3–4
	Topotecan	Approx. 3	>10
	Irinotecan	Approx. 16	>100

^a Effective range is defined as the minimal drug dose causing 50% tumour regression relative to the MTD in mice.

33 ± 15 ng/ml per h and 50 ± 18 ng/l per h on days 1 and 5, respectively. In Fig. 1 the systemic exposure achieved in mice (at 7 mg/kg, equivalent to approximately 22 mg/M²) is superimposed upon clinical results obtained in the phase I trial of Irofulven. An important consideration when comparing systemic exposures for Irofulven across species is the contribution of protein binding. The *in vitro* serum protein binding shows that Irofulven is 80–82% bound in mice and 51–52% bound in man (over a concentration range from 500 to 1000 ng/ml). Although a slight difference in protein binding exists between species, it cannot account for the greater than 6-fold difference in plasma systemic exposure between that required for even minimal antitumour effect and that tolerated in the adult phase I study. These results help in understanding the 'disconnect' often observed between results from preclinical testing and clinical activity. Comparing those systemic exposures that yield significant antitumour activity against human cancers in mice with systemic drug exposure at the MTD in patients may identify, at a relatively early stage in development, those agents that will ultimately fail in the clinic. While such data are not available before testing in man, such 'retrotranslation'¹ can be rather useful in making informed decisions about advancing an agent to phase II testing.

3.1. Predicting human pharmacology

As described above, perhaps the greatest challenge in achieving relative uniformity between the research conditions present in the preclinical and clinical settings is accounting for the pharmacokinetic differences between mouse and man. The conventional approach is to evaluate the absorption, distribution, metabolism and excretion (ADME) properties to select compounds that have acceptable pharmacokinetic properties. Because of the complexity of factors involved in ADME, several approaches have been proposed to develop accurate methods for predicting the human pharmacology of an agent. This prediction is particularly important for agents that demonstrate marked interspecies differences in their pharmacokinetics. Conventional allometric scaling (CAS) is the most commonly used technique for predicting human pharmacokinetic variables [20]. Nomura and colleagues have applied these principles to calculating the clinically effective doses (CED) of anticancer agents in the context of human xenograft experiments [21]. An example of their studies using mitomycin C is shown in Fig. 2. Based upon rates of drug clearance and the toxic doses in several species, they calculated the CED. Allometric scaling has provided useful information for a number of compounds

Table 3
Antitumour activity of Irofulven against childhood tumour xenografts

Tumour	Dose (mg/kg)				
	7.0	4.6	3.0	2.0	1.3 ^a
DAOY	+++++	+++++	+++++	+++++	++
D283	+++++	+++++	+++++	++	++
SJ-BT12	ND	++++	+/– ^a	– ^a	– ^a
SJ-BT16	++	++++	++	++	++
SJ-BT27	ND	+++++	+++++	++	+
SJ-BT29	+++++	++	+++ ^a	ND	ND
SJ-BT33	– ^a	– ^a	– ^a	– ^a	– ^a
SJ-BT34	+++++	+++++	+++++	ND	ND
SJ-BT36	+++++	+++++	++++	++	++ ^a
SJ-BT37	+++++	++++	++	++	– ^a
SJ-BT40	++++	++++	++ ^a	++	– ^a
SJ-GBM2	+++++	+++++	++	ND	ND
NB-1771	ND	ND	+	– ^a	– ^a
NB-1382	++++	++++	+++ ^a	ND	ND
NB-1643	+++++	+++++	+++	ND	ND
NB-1691	+++++	++	++	++	ND
Rh18	+++++	++++	++++	++	++
Rh28	+++++	+++++	+++++	+++++	++
Rh30	ND	ND	ND	+++++	+++++
Rh36	+++	++	++	++	– ^a
CR + PR/Total	14/16	14/18	8/19	3/16	1/14

Response criteria: –, no growth inhibition; +, growth inhibition equals one tumour-volume doubling time; ++, growth inhibition equals two tumour-volume doubling times; +++, growth stasis; +++++, partial response (≥0% regression; PR); +++++, complete response (CR) with regrowth by week 12; +++++, CR maintained at week 12 (no regrowth by week 12); ND, not determined.

^a Growth inhibition not significant ($P > 0.05$); all other results were significantly different from controls ($P < 0.05$).

¹ A term coined by Scott Kaufmann at Mayo Clinic to describe such studies.

that are metabolised and eliminated via the kidney [22]. The method is less robust for agents whose elimination is due to active transport in the liver or kidney. Such agents generally show large variations in interspecies pharmacokinetic properties. Newer methods incorporating additional scaling factors such as time normalisation, protein binding, brain weight and liver conjugation activity have been proposed [23]. For drugs eliminated by liver metabolism alone or by metabolism and elimination in the bile, other factors such as bile flow rates and *in vitro* microsome or hepatocyte metabolism have been used to normalise the *in vivo* clearance and have improved the prediction of their *in vivo* human clearances. An alternative approach to modelling, termed physiologically based pharmacokinetics (PBPK), has been proposed recently by Poulin and Thiele [24]. This model allows prediction of disposition profiles based on the physicochemical and biochemical properties of the drug combined with the species-specific physiological characteristics. The value of PBPK prediction is highly dependent on input variables such as tissue:plasma partition coefficients and *in vivo* blood clearance. A comparison of the predictive value of PBPK using the antimicrobial diaminopyrimidine, epiroprim, an agent that has marked species differences in its elimination pathways, has recently been reported [23]. The best prediction of human pharmacokinetics was made by using the tissue composition model to predict tissue:plasma partition coefficients, and allometric scaling of the animal's intrinsic *in vivo* blood clearance normalised by the ratios of animal:human intrinsic clearances determined *in vitro* with hepatocytes. We are unaware of similar studies with anticancer drugs, but it may be of interest to test the predictive value of the

models using some of the 'failed' antitumour agents discussed above.

3.2. Molecular-target inhibition

The discussion above on the role of pharmacokinetic modelling, and the 'disconnect' between preclinical and clinical results, is based largely on our experiences with classical cytotoxic agents. However, there is an increasing focus on developing novel agents that target specific molecules that drive the transformed phenotype. The now 'classic' example is Imatinib, a 2-phenylamino-pyrimidine that inhibits the Bcr/Abl kinase in chronic myelogenous leukaemia, and *c-kit* kinase in gastrointestinal stromal tumours. In developing agents against very specific targets it is a reasonable concern whether conventional xenograft models will be valuable for identifying and prioritising such compounds for clinical development, or whether this is the role of genetically engineered mouse models? Clearly, where a tumour is 'driven' by a single genetic change (i.e. overexpression of a receptor as in *HER2* amplification, or mutation leading to constitutive activation of a tyrosine kinase as in the case of Bcr/Abl) there should be an absolute correlation between target inhibition and biological response. For example when induced by doxycycline, transgenic MMTV-rTA/TetO-NeuNT mice develop multiple invasive mammary carcinomas that regress completely following abrogation of Neu expression [25]. In other cases, such as overexpression of *ERBB1*, there is less 'coupling' between target inhibition and the biological response of human tumours (e.g. the effect of Gefitinib). Thus, although genetically engineered models may be very useful for testing the ability of a

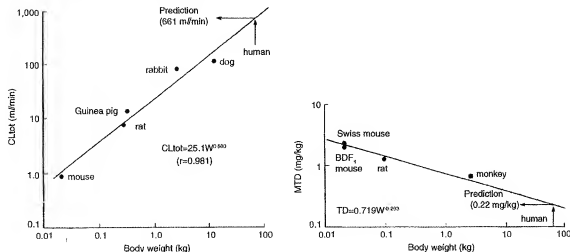


Fig. 2. Use of allometric scaling to estimate the clinically effective dose (CED) for mitomycin C. The CED is calculated as the human AUC \times mouse CL₁₀₀. The left panel demonstrates clearance against body weight for various species. The right panel plots the maximum tolerated dose (MTD) as a function of body weight in different species. (From Nomura *et al.* [21]. Figure originally published in [31]. Mordenti J. Dosage regimen design for pharmaceutical studies conducted in animals. *J Pharm Sci* 1986, 75, 852–857. Reproduced with permission of John Wiley & Sons, Inc. Copyright © 1986 Wiley-Liss, Inc., a Wiley Company. URL: <http://www3.interscience.wiley.com/cgi-bin/jabout/68503813/ProductInformation.htm>).

compound to inhibit its putative molecular target, they do not necessarily predict that the target itself will be coupled to the biological 'read-out' in real human cancers. Indeed, the value of the xenograft model might be that such 'coupling' information could be obtained at an early stage in preclinical development. One can extend the principles of relating pharmacokinetics to tumour response to encompass target inhibition in the model system. These experiments, which require sampling of tumour tissue at multiple time points, are used to construct a pharmacodynamic model that relates the amount and duration of target inhibition to the tumour response. This approach has been used extensively in the development of the MEK1 inhibitor PD184352 [26]. Such studies can be extended to establish a correlation between target inhibition and drug systemic exposure in the mouse model, and potentially allow for predicting both dose and frequency of drug administration in patients. Of essence, however, is that the models used should accurately parallel the metabolic characteristics of human cancers with respect to target expression and dependence. Whether the models commonly used for drug development meet these criteria is less well established. Most commonly used models in drug development have been grown in cell culture for many years and may have deviated significantly from the original human cancer. Our experience with rhabdomyosarcoma cell lines suggests that significant changes occur with serial passaging *in vitro*, and indeed tumours established from such *in vitro* cultured lines have very different drug sensitivities from those of the 'parental' tumours established directly from biopsies and maintained in mice. In general our studies have limited the use of tumours to the first 30 serial passages in mice in an attempt to limit such 'drift'. However, it should now be possible to determine more accurately whether the model is representative of the original tumour, and how long it can be passaged in mice before it deviates significantly. A recent initiative by the NCI/ Cancer Treatment Evaluation Program (CTEP) and the Children's Oncology Group aims to characterise the available models of childhood tumours through gene-expression and proteomics profiling (Pediatric oncology preclinical protein tissue array project; POPP-TAP). These data will be of value in establishing whether tumours grown as xenografts maintain molecular characteristics that will be essential for their use in developing so-called molecularly targeted therapies. Where we have access to the original tumour we have started to compare expression profiles between the biopsy and early-passage xenograft tumours. Initial results (J. Dome, P. Houghton, data not shown) with Wilms' tumours look promising in that there is a very high correlation between primary tumour and xenograft, particularly for more highly expressed genes. This technique may be very useful for monitoring changes in gene expression with continuous passage of

tumour in mice, and for setting criteria that allow us to determine the number of passages over which a particular model remains valid.

3.3. Application of preclinical *in vivo* models to childhood cancer

Developing new therapies for childhood solid tumours has certain constraints that are seldom encountered with the neoplastic diseases of adults. Childhood tumours are rare, with about 12 500 new cases in patients less than 21 years old each year in the United States; hence, the numbers of children with a particular diagnosis at any one institution are usually not adequate for large-scale drug evaluation or randomised clinical trials. For example, of the new phase I agents evaluated in adult malignancies, less than 30% receive adequate evaluation in children. Furthermore, the NCI drug-screening strategy focuses on the selection of new anticancer agents with specific activity against adult neoplastic diseases (e.g. colon, lung, breast, prostate etc.), so that agents with specific activity against childhood malignancies may not be identified. Many common solid tumours of childhood respond to drugs of established efficacy, resulting in cure for a substantial number of patients. This ethically precludes the use of 'experimental' agents in many untreated cases. However, over the last decade, survival rates for patients with disseminated tumours at diagnosis have improved only slightly, if at all. This lack of progress must be attributed in part to the slow rate at which new active compounds reach the clinic, and the failure to integrate laboratory and clinical efforts in a way that will generate a steady flow of promising experimental leads that can be used in the design of productive approaches to treatment. The results obtained using the xenograft models of childhood tumours suggest that the integration of preclinical pharmacology and the rational use of such tumour models could provide a system for identifying, and prioritising, novel agents for clinical trials in children. However, a systematic approach has yet to be established.

To address this problem, NCI/CTEP initiated a series of meetings that resulted in a consensus document [27] detailing the available models of childhood tumours. These models included human xenografts, syngeneic rodent tumours and genetically engineered (knockout and transgenic) mice that would be available for a national effort to screen new agents. A tentative plan for evaluating new agents that incorporates the principles of integrating pharmacokinetic and pharmacodynamic studies discussed above is presented in Fig. 3. In this schema, drugs that are close to entering clinical trials, or are in early-stage clinical development (phase I in adults) will be screened against panels of childhood cancers including the more frequently occurring solid

tumours and acute lymphocytic leukaemia. In this proposal, new agents are screened initially at the MTD in each panel comprising 6–10 independently derived xenografts representing a tumour type (e.g. neuroblastoma). Where available a transgenic model will be included. For example the targeted overexpression of the human *MYCN* proto-oncogene to the neural crest of C57B6/J \times Balb/c or 129X1/SvJ mice predisposes the animals to develop neuroblastoma with high penetrance [28]. Similarly, models of rhabdomyosarcoma [29] and medulloblastoma [30] have been developed and may be utilised in the screening. Where the agent demonstrates significant activity (i.e. regression for an agent expected to be cytotoxic, and <25% tumour growth for a cytostatic), a full dose–response correlation together with pharmacokinetic and, where appropriate, pharmacodynamic studies will be undertaken. For agents that inhibit signal transduction, pharmacodynamic studies may be necessary even in the absence of significant antitumour activity, as it will be important to establish that drug exposures in the mouse are indeed adequate to inhibit the drug target. The approach presented in Fig. 3 reflects much of the uncertainty over the development of ‘molecularly targeted’ agents. That is, should we evaluate the agent only against tumours expressing the target, or do we ‘assume’ that the agent may have numerous

targets (e.g. most tyrosine kinase inhibitors). Hopefully, the POPP-TAP initiative will ultimately catalogue ‘molecular targets’ in these tumours and have the potential to identify activated pathways that would allow ‘matching’ of drug to particular tumour models. Should we test such agents at a dose that gives a biological response (i.e. target inhibition) or at the MTD? The simplest approach is to test at the MTD, irrespective of the drug mechanism. The ‘up side’ of this is that it reduces the number of false-negative results, but potentially will increase the number of false positives. For example, the mouse may be highly tolerant of an agent, so allowing high systemic exposure to the drug and revealing antitumour activities independent of the primary molecular target. If such exposures cannot be achieved in man, then it is likely that any antitumour activity determined in the models will not be reproduced in the clinic.

In summary, when antitumour activity in preclinical *in vivo* models of childhood cancer is integrated with pharmacological studies, there appears reason to be optimistic about the predictive value of such experiments. While one may have reason to be optimistic, one should also be cautious and not extrapolate beyond the real use of any model system. The project outlined in Fig. 3, if it is initiated, will be an interesting experiment

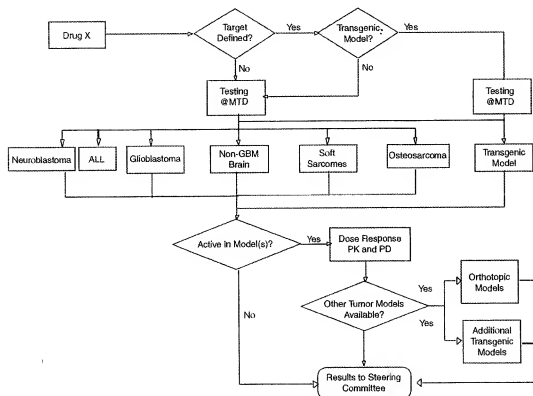


Fig. 3. Schematic representation of a potential preclinical testing programme (redrawn from Houghton and colleagues [27], and used with permission from the American Association for Cancer Research Inc). ALL, acute lymphocytic leukaemia; MTD, maximum tolerated dose; PD, Pharmacodynamic; PK pharmacokinetic.

that will test the value of integrating pharmacokinetic and pharmacodynamic studies with antitumor testing as a paradigm for drug development, particularly for rare cancers.

Acknowledgements

We thank the numerous research technologists, post-doctoral fellows and colleagues at St. Jude who have contributed to work summarised in this article, which was conducted over the last 25 years. These studies have been supported through PHS award CA23099 from the National Cancer Institute, and by the American, Lebanese, Syrian, Associated Charities (ALSAC).

References

- Johnson JI, Decker S, Zaharevitz D, et al. Relationships between drug activity in NCI preclinical in vitro and in vivo models and early clinical trials. *Br J Cancer* 2001, **84**, 1424–1431.
- Heterotransplantation of human adenocarcinomas of the colon and rectum to the mouse mutant Nude. A study of nine consecutive transplantations. *Acta Pathol Microbiol Scand [A]* 1971, **79**:159–169.
- Rygaard J, Povlsen CO. Heterotransplantation of a human malignant tumour to "Nude" mice. *Acta Pathol Microbiol Scand* 1969, **77**, 758–760.
- Lock RB, Liem N, Farnsworth ML, et al. The nonobese diabetic/severe combined immunodeficient (NOD/SCID) mouse model of childhood acute lymphoblastic leukemia reveals intrinsic differences in biologic characteristics at diagnosis and relapse. *Blood* 2002, **99**, 4100–4108.
- Houghton JA, Cook RL, Lutz PJ, Houghton PJ. Childhood rhabdomyosarcoma xenografts: responses to DNA-interacting agents and agents used in current clinical therapy. *Eur J Cancer Clin Oncol* 1984, **20**, 955–960.
- Houghton PJ, Horton JK, Houghton JA. Drug sensitivity and resistance in the xenograft model. In: Maurer HM, Rynman FB, Poebch C, eds. *Rhabdomyosarcoma and Related Tumors in Children and Adolescents*. Boca Raton, CRC Press, 1991, 187–202.
- Houghton JA, Cook RL, Lutz PJ, Houghton PJ. Melphalan: a potential new agent in the treatment of childhood rhabdomyosarcoma. *Cancer Treat Rep* 1985, **69**, 91–96.
- Horowitz ME, Etchubanas E, Christensen ML, et al. Phase II testing of melphalan in children with newly diagnosed rhabdomyosarcoma: a model for anticancer drug development. *J Clin Oncol* 1988, **6**, 308–314.
- Houghton PJ, Cheshire PJ, Hallman 3rd JC. Evaluation of a novel bis-naphthalimide anticancer agent, DMP 840, against human xenografts derived from adult, juvenile, and pediatric cancers. *Cancer Chemother Pharmacol* 1994, **33**, 265–272.
- Houghton PJ, Cheshire PJ, Hallman 2nd JD, Houghton JA. Therapeutic efficacy of the cyclopropylpyrrolidinone, carzelesin, against xenografts derived from adult and childhood solid tumors. *Cancer Chemother Pharmacol* 1995, **36**, 45–52.
- Houghton PJ, Houghton JA, Myers L, Cheshire P, Howbert JJ, Grindley GB. Evaluation of N-(5-indanylsulfonyl)-N'-(4-chlorophenyl)-urea against xenografts of pediatric rhabdomyosarcoma. *Cancer Chemother Pharmacol* 1989, **25**, 84–88.
- Pappo AS, Lyden E, Breneman J, et al. Up-front window trial of topotecan in previously untreated children and adolescents with metastatic rhabdomyosarcoma: an intergroup rhabdomyosarcoma study. *J Clin Oncol* 2001, **19**, 213–219.
- Furman WL, Stewart CF, Poquette CA, et al. Direct translation of a protracted irinotecan schedule from a xenograft model to a phase I trial in children. *J Clin Oncol* 1999, **17**, 1815–1824.
- Cosetti M, Wexler LH, Calleja E, et al. Irinotecan for pediatric solid tumors: the Memorial Sloan-Kettering experience. *J Pediatr Hematol Oncol* 2002, **24**, 101–105.
- Santana VM, Zamboni WC, Kirstein MN, et al. A pilot study of protracted topotecan dosing using a pharmacokinetically guided dosing approach in children with solid tumors. *Clin Cancer Res* 2003, **9**, 633–640.
- Zamboni WC, Stewart CF, Thompson J, et al. Relationship between topotecan systemic exposure and tumor response in human neuroblastoma xenografts. *J Natl Cancer Inst* 1998, **90**, 505–511.
- Santana VM, Zamboni WC, Gajjar A, et al. Pharmacokinetically-guided use of topotecan (TPT), given (daily \times 5) \times 2, in children with relapsed solid tumors. *Proc Annu Meet Am Soc Clin Oncol* 1997, **16**, 1839A.
- Leggass M, Stewart CF, Woo MH, et al. Relation between Irinotecan (MGI-114) systemic exposure and tumor response in human solid tumor xenografts. *Clin Cancer Res* 2002, **8**, 3000–3007.
- Eckhardt SG, Baker SD, Britten CD, et al. Phase I and pharmacokinetic study of irinotecan, a novel mushroom-derived cytotoxin, administered for five consecutive days every four weeks in patients with advanced solid malignancies. *J Clin Oncol* 2000, **18**, 4086–4097.
- Ritschel WA, Vachharajani NN, Johnson RD, Hussain AS. The allometric approach for interspecies scaling of pharmacokinetic parameters. *Comp Biochem Physiol C* 1992, **103**, 249–253.
- Nomura T, Sakurai Y, Inaba M. *The Nude Mouse and Anticancer Drug Evaluation*. Tokyo, Japanese Journal of Cancer and Chemotherapy Publishers Inc, 1996.
- Lave T, Portmann R, Schenker G, et al. Interspecies pharmacokinetic comparisons and allometric scaling of napsagatran, a low molecular weight thrombin inhibitor. *J Pharm Pharmacol* 1999, **51**, 85–91.
- Luttringer O, Theil FP, Poulin P, Schmitt-Hoffmann AH, Guentert TW, Lave T. Physiologically based pharmacokinetic (PBPK) modeling of disposition of epiropram in humans. *J Pharm Sci* 2003, **92**, 1990–2007.
- Poulin P, Theil FP. Prediction of pharmacokinetics prior to in vivo studies. I. Mechanism-based prediction of volume of distribution. *J Pharm Sci* 2002, **91**, 129–156.
- Moody SE, Sarkisian CJ, Hahn KT, et al. Conditional activation of Neu in the mammary epithelium of transgenic mice results in reversible pulmonary metastasis. *Cancer Cell* 2002, **2**, 451–461.
- Scholt-Leopold JS, Dudley DT, Herrera R, et al. Blockade of the MAP kinase pathway suppresses growth of colon tumors in vivo. *Nat Med* 1999, **5**, 810–816.
- Houghton PJ, Adamson PC, Blaney S, et al. Testing of new agents in childhood cancer preclinical models: meeting summary. *Clin Cancer Res* 2002, **8**, 3646–3657.
- Weiss WA, Aldape K, Mohapatra G, Feuerstein BG, Bishop JM. Targeted expression of MYCN causes neuroblastoma in transgenic mice. *EMBO J* 1997, **16**, 2985–2995.
- Sharp R, Recio JA, Jhappan C, et al. Synergism between INK4a/ARF inactivation and aberrant HGF/SF signaling in rhabdomyosarcomagenesis. *Nat Med* 2002, **8**, 1276–1280.
- Wetmore C, Eberhart DE, Curran T. Loss of p53 but not ARF accelerates medulloblastoma in mice heterozygous for patched. *Cancer Res* 2001, **61**, 513–516.
- Mordenti J. Dosage regimen design for pharmaceutical studies conducted in animals. *J Pharm Sci* 1986, **75**, 852–857.

The Oncolytic Effect of Recombinant Vesicular Stomatitis Virus Is Enhanced by Expression of the Fusion Cytosine Deaminase/Uracil Phosphoribosyltransferase Suicide Gene

Mercedes Porosnicu, Abdul Mian, and Glen N. Barber

Department of Microbiology and Immunology and Sylvester Comprehensive Cancer Center, University of Miami School of Medicine, Miami, Florida

ABSTRACT

Vesicular stomatitis virus (VSV) has recently been demonstrated to exhibit significant oncolytic capabilities against a wide variety of tumor models *in vitro* and *in vivo*. To potentially enhance the oncolytic effect, we generated a novel recombinant VSV (rVSV) that expressed the fusion suicide gene *Escherichia coli* cytosine deaminase (CD)/uracil phosphoribosyltransferase (UPRT). rVSV encoding the CD/UPRT fusion gene (VSV-C:U) exhibited normal growth properties and generated high levels of biologically active CD/UPRT that could catalyze the modification of 5-fluorocytosine into chemotherapeutic 5-fluorouracil (5-FU), which exhibited considerable bystander effect. Intratumoral inoculation of VSV-C:U in the presence of the systemically administered prodrug 5-fluorocytosine produced statistically significant reductions in the malignant growth of syngenic lymphoma (A20) or mammary carcinoma (TSA) in BALB/c mice compared with rVSV treatments or with control 5-FU alone. Aside from detecting prolonged therapeutic levels of 5-FU in VSV-C:U-treated animals harboring TSA tumors and enhancing bystander killing of tumor cells, we demonstrated marked activation of IFN- γ -secreting cytotoxic T cells by enzyme-linked immunosorbent assay that may have also facilitated tumor killing. In conclusion, the insertion of the fusion CD/UPRT suicide gene potentiates the oncolytic efficiency of VSV by generating a strong bystander effect and by contributing to the activation of the immune system against the tumor without detrimentally altering the kinetics of virus-mediated oncolysis and may be useful in the treatment of malignant disease.

INTRODUCTION

The delivery of suicide genes into malignant cells affords the opportunity of increasing the efficacy of tumor eradication while limiting toxic side effects that are often found with systemic chemotherapy (1). Suicide gene therapy involves the transduction of cancer cells with a nonmammalian gene encoding an enzyme that converts a relatively innocuous, systemically delivered prodrug into a highly toxic chemotherapeutic (1). However, a major issue when considering enzyme-prodrug systems for the treatment of cancer remains the effective transport of the suicide gene into the appropriate tumor tissue. Although a number of strategies have been used to target tumor cells effectively, including the use of replication-defective virus vectors, problems associated with low levels of gene transfer and expression are encountered frequently (2).

Recently, we genetically modified a replication-competent oncolytic virus, VSV,¹ to carry foreign genes such as cytokines (3). We

reasoned that this approach could improve tumor cell killing by combining the effect of direct viral oncolytic activity with the augmented antitumor immune responses or, in the case of suicide genes, bystander cytotoxic action. VSV is an enveloped, negative-stranded RNA virus with a simple genetic structure of five genes and well-characterized immunobiology (4). VSV is relatively innocuous in humans, inducing at the most a flu-like syndrome (5). However, the majority of the population lacks antiviral antibodies that could conceivably limit virus treatment, and in general, the incidence of exposure to the VSV is low (6). VSV is a nonintegrating virus that replicates in the cytoplasm, does not undergo genetic reassortment, and has no known transforming potential (4). Evidence indicates that the mechanism of VSV-induced oncolysis involves taking advantage of defects in the IFN host defense system prevalent in tumor cells. Lack of an effective IFN response allows propagation of virus, leading to rapid cytolysis (7, 8). Our attempts at improving the efficacy of VSV tumor therapy have indicated that rVSV is easy to generate and is amenable to high-titer production and purification (3). rVSV variants generated thus far have also demonstrated greater oncolytic activity than the WT virus in tumor therapy studies and appear greatly attenuated *in vivo* compared with the WT parent virus (3).

CD is a well-characterized enzyme-prodrug system that functions similar to the herpesvirus TK paradigm (9, 10). However, CD is an enzyme found in bacteria and fungi and not mammalian cells that can catalyze the hydrolytic deamination of the relatively nontoxic antifungal agent 5-FC to the commonly used chemotherapeutic 5-FU. 5-FU can be further modified by cellular enzymes into potent pyrimidine antimetabolites, which induce cell death by inhibiting thymidine synthetase and DNA and RNA synthesis (11). 5-FU has also been reported to diffuse freely through cellular membranes to generate a strong direct bystander effect (12). Reports using human colorectal xenograft murine models indicated that transfection of 4% of the tumor cells with *Escherichia coli* CD gene led to survival rates of 60% (13). However, limited success has been reported in several experiments that used selected breast and pancreatic tumor models because such cells were found to be relatively resistant to 5-FU, possibly attributable to defects in downstream cellular pathways that are responsible for the metabolism of this enzyme (14, 15). In support of this hypothesis, expression of bacterial or yeast UPRT in mammalian cells was found to greatly enhance the sensitivity of the cells to 5-FU (15, 16). UPRT is an enzyme that catalyzes the conversion of uracil and 5-FU directly into UMP and 5-fluoro-UMP, respectively, by bypassing rate-limiting reactions controlled by the cellular enzymes. Coexpression of CD and UPRT has been reported to increase the sensitivity to 5-FC 10–168 times when compared with CD alone, depending on the experimental model used (15–17).

To examine whether the CD/5-FC system could be used with rVSV as a vector delivery system, we generated a novel rVSV suicide gene model expressing the *E. coli* CD/UPRT fusion gene (rVSV-C:U). We show here that the expression of the foreign gene does not detrimentally affect VSV replication and results in the generation of high levels of CD and UPRT enzyme activity after infection of the cell. Importantly, rVSV-C:U retained selective oncolytic activity and displayed

Received 6/2/03; revised 8/4/03; accepted 9/15/03.

Grant support: Mercedes Porosnicu was supported in part by 2001 American Society of Clinical Oncology Young Investigator Award and 2001 AACR-Bristol Myers Squibb Clinical Research Fellowship.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Glen N. Barber, Room 514, Papanicolaou Building, 1550 North West 10th Avenue (M710), University of Miami School of Medicine, Miami, Florida 33136. Phone: (305) 243-5914; Fax: (305) 243-5885; E-mail: gbarber@mimed.miami.edu.

Abbreviations used: VSV, vesicular stomatitis virus; rVSV, recombinant VSV; CD, cytosine deaminase; UPRT, uracil phosphoribosyltransferase; 5-FC, 5-fluorocytosine; 5-FU, 5-fluorouracil; TK, thymidine kinase; HPLC, high performance liquid chromatography; GFP, green fluorescent protein; IFN, human IFN; TLC, TLC sheet; BHK, baby hamster kidney; MOI, multiplicity of infection; WT, wild-type; HI-VSV, heat-inactivated VSV; GCV, ganciclovir; ELISPOT, enzyme-linked immunospot; CPE, cytopathic effect.

effective bystander effect *in vivo* and *in vitro* with concomitant tumor cell killing. Our data indicate that incorporating CD-based strategies into rVSV-mediated gene therapy protocols may improve the oncologic potential of this tumor treatment approach.

MATERIALS AND METHODS

Chemicals. [³H]Cytosine (9.5 Ci/mmol), [2-¹⁴C]uracil (54 mCi/mmol), 5-phosphoribosyl-1-pyrophosphate, uracil, cytosine, UMP, 5-FC, low EEO type 1 agarose, and 2,3 5-bromo-4-chloro-3-indolyl phosphate were purchased from Sigma Chemical Co. (St. Louis, MO), and streptavidin-alkaline phosphatase was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). hIFN- α 2a and gancyclovir were obtained from Roche Laboratories, Inc. (Nutley, NJ), and 5-FU was purchased from Pharmacia & Upjohn Co. (Kalamazoo, MI). Antimouse IFN- γ antibodies and biotinylated anti-IFN- γ monoclonal antibodies were acquired from Pharmingen (San Diego, CA). TLCs and polyethyleneimine-impregnated TLCs were acquired from EM Science (Gibbstown, NJ), and Nunc MaxiSorp plates were obtained from Nunc (Naperville, IL).

Cell Culture. Human multiple myeloma cell line 8226/Dox40 was a gift from Dr. L. H. Boie (University of Miami; Ref. 18), and the murine T-cell lymphoma EL4 and the murine B-cell lymphoma A20 were gifts from Dr. J. Rosenblatt (University of Miami; Refs. 19 and 20). The TSA mammary adenocarcinoma cell line was a gift from Dr. A. Rakhmievich (University of Wisconsin, Madison, WI; Ref. 2). B16(F10) melanoma cells, 293T embryonic kidney cells, and the BHK cell line BHK-21 were purchased from American Type Culture Collection (Manassas, VA). HMVEC, a human microvascular endothelial cell line, was acquired from Clonetics Corp. (San Diego, CA). Cells were grown in suspension or in monolayer in the recommended culture medium with fetal bovine serum.

Construction of Recombinant Virus. The recovery of the infectious recombinant viruses and the growth, purification, concentration, and titration of the WT and recombinant viruses were performed according to the methods described previously (21, 22). The C:U gene was PCR amplified from the pORF-codA:upp expression vector (InvivoGen). The primers were 5'-GAAGCTCGAGCACTATGGTGTGCAATACGCTT and 3'-GTGCGAGC-TAGCGAATTTCCACAGAGTTATTCCTGATAC, introducing *XhoI* and *NheI* restriction sites, respectively. The C:U cDNA was then excised from the amplification product with *XhoI* and *NheI* and cloned into the *XhoI/NheI* site of pVSV-XN2 (23) using a Zero Blunt TOPO shuttle vector (Invitrogen, Carlsbad, CA). The plasmid pVSV-XN2 contains the entire VSV genome and has unique *XhoI* and *NheI* sites flanked by VSV transcription start and stop signals. After recovery of the infectious rVSV in BHK cells, monoclonal virus was obtained by plaque purification and further purification, and concentration was achieved by sucrose centrifugation. Virus stock titers were measured by standard plaque assay.

CD Enzyme Assay Measured Spectrophotometrically. CD activity was measured directly by using cell lysates or indirectly by measuring 5-FU released in the culture media. BHK cells (5×10^6) were infected with rVSV-GFP at MOI = 5.0, rVSV-C:U at MOI = 0.1, or rVSV-C:U at MOI = 5.0. To measure 5-FU in culture media, 5 mM 5-FC was added to the media 2 h postinfection. Samples of culture media (10 μ l) were collected at different time points and quenched with 990 μ l of 0.1 M HCl. To measure C:U activity using cell lysates, 6 h postinfection cells were washed twice with PBS, resuspended in PBS at a concentration of 5×10^6 cells/100 μ l PBS, and exposed to five cycles of thawing/freezing. Fifty μ l of each sample were mixed with 950 μ l of 3 M solution of 5-FC in PBS and incubated at 37°C. At different time points, 50- μ l samples were collected and quenched with 950 μ l of 0.1 M HCl (24).

The concentration of 5-FU and 5-FC for each condition was determined spectrophotometrically using the following formulas: 5-FC (mM) = $0.119(A_{290}) - 0.025(A_{225})$; and 5-FU (mM) = $0.185(A_{225}) - 0.049(A_{290})$. Each experiment was performed independently three times.

CD and UPR Chromatographic Assays. CD and UPR enzymatic activities were measured as described previously (14, 15, 25). 293T cells were transfected with C:U gene using the LipofectAMINE system. Confluent BHK cells were infected with rVSV-C:U at MOI = 0.1 or MOI = 5.0. For CD activity, 6 h postinfection BHK-infected cells, 293T-transfected cells, and

293T and BHK control cells were harvested in a mixture of 100 mM Tris (pH 7.5), 1 mM EDTA, and 1 mM DTT at a concentration of 1×10^6 cells/10 μ l lysis buffer and frozen. At the time of enzymatic measurements, cells had gone through five freeze/thaw cycles, and cellular debris was removed by centrifugation (5 min at 14,000 rpm). For UPR activity, BHK-infected cells (6 h postinfection) or control BHK cells were harvested in lysis buffer [50 mM Tris HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 mM DTT, and 1% Triton X-100] at a concentration of 1×10^6 cells/10 μ l lysis buffer and incubated for 30 min at 4°C. Ten μ l of cell lysate were mixed with 10 μ l of [³H]cytosine (0.5 μ Ci) for CD activity or with 10 μ l of reaction buffer [100 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 10 mM 5-phosphoribosyl-1-pyrophosphate, and 1 μ l of uracil-5,6-³H (1 μ Ci)] for UPR activity and incubated at 37°C for 2 h. Ten μ l of each reaction and 10 μ l of a marker solution (unlabeled cytosine and uracil or uracil and UMP) were loaded on TLCs for CD activity or on propidium iodide-TLCs for UPR activity and chromatographed in butanol/water (86:14). The bands corresponding to cytosine and uracil or to uracil and UMP were cut under short-wave UV illumination, and the radioactivity was assayed by liquid scintillation counter. Each experiment was performed independently three times.

Kinetics of Cytolytic VSV and One-Step Growth Curve. BHK-21 cells were infected with WT-VSV, rVSV-GFP, rVSV-TK, or rVSV-C:U at MOI = 10. After 45 min, infection media were removed; cells were washed five times with PBS and covered with fresh media. At the indicated time points, samples of culture media were collected, and viral titers were measured by standard plaque assay (5). For analysis of virus replication and cytolysis after infection with low-dose virus, BHK cells were plated at 10^5 cells/well in 6-well dishes and infected 18 h later with MOI = 0.01 of WT-VSV, rVSV-GFP, rVSV-TK, or rVSV-C:U for 45 min, washed once with PBS, and covered with fresh media. At 12, 18, 24, and 28 h, cells were examined by microscopy. At 24 and 48 h, cell viability was assayed by trypan blue exclusion, culture media samples were collected, and viral titers were measured by standard plaque assay.

In Vitro Evaluation of the Effect of 5-FU Bioactivation and Effect of 5-FU and 5-FC on Virus Replication. TSA or BHK cells were plated at a density of 5×10^5 cells in 6-well dishes and treated (in triplicate) with different concentrations of 5-FU or 5-FC (1, 3, 6, and 10 μ M and mM, respectively). After 24 h, cells were infected with HI-VSV or rVSV-GFP at MOI = 0.1 or 1.0. The percentage of infected cells was evaluated at 4, 12, and 24 h by fluorescent microscopy. Samples of culture media collected at 24 h were used to measure viral titers by standard plaque assay. Cell survival was measured by trypan blue staining at 24 h. A20 cells were infected with HI-VSV, rVSV-GFP, or rVSV-C:U at MOI = 0.1. After 4 h, infected cells were mixed in different proportions with noninfected cells at a total concentration of 5×10^5 cells/well in 12-well dishes, and 1 mM 5-FC was added to the media. Cell survival was evaluated by trypan blue staining after 48 h. HMVEC, A20, EL4, 8226/Dox40, or TSA cells were infected with rVSV-C:U at MOI = 0.1 or mock infected (HI-VSV) in the presence or absence of 1 or 3 mM 5-FC (in the case of A20 cells). Samples of culture media from each condition were collected after 24 h and incubated at 60°C for 10 min to inactivate the virus and to preserve the 5-FU activity (26). Aliquots of treated culture media were mixed in different ratios with corresponding fresh media and added to cells freshly plated at a density of 2×10^5 cells/well in 6-well plates. Cell survival was assayed by trypan blue exclusion after 96 h. Complete inactivation of VSV by heating at 60°C was checked by standard plaque assay.

Virus/Suicide Gene System-Induced Cell Killing. HMVEC, TSA, B16(F10), 8226/Dox40, or EL4 cells were treated with rVSV-C:U at MOI = 0.1, in the presence or absence of 3 mM 5-FC. Cells treated with 5-FC alone, with HI-VSV, or with WT-VSV at MOI = 0.1 were used as control. Cell survival was evaluated by trypan blue exclusion after 18 h.

IFN Protection Assay. HMVEC or 8226/Dox40 cells were cultured in duplicate in 6-well dishes at a density of 5×10^5 cells/well, and 12 h later, hIFN- α (1000 U/ml) was added to the culture media in half of the dishes. After 24 h, culture media were removed, and hIFN- α -treated cells and untreated cells were infected with WT-VSV, rVSV-GFP, rVSV-TK, or rVSV-C:U at MOI = 0.1. Postinfection samples of culture media (24 h) were collected for viral titers, and cell viability was determined by trypan blue exclusion. A20 or control EL4 cells were infected with rVSV-GFP at MOI = 0.1, 1.0, or 100 and then cultured in 25-cm flasks at a density of 10^6 cells/ml. Postinfection cells (4, 8, 24, and 48 h) were resuspended, and samples

were analyzed by fluorescent microscopy and flow cytometry for calculation of the percentage of GFP-positive cells. A20 cells were infected with rVSV-C:U at MOI = 0.1 in the presence or absence of 1 mM 5-FC. Control cells were treated with 5-FC alone, HI-VSV, or infected with rVSV-TK in the presence of 10 μ g/ml GCV or with WT-VSV at MOI = 0.1. Treated cells were cultured at a density of 5×10^5 cells/ml in 25-cm flasks. At 24, 48, and 72 h postinfection, cells were resuspended, and samples were collected for cell survival evaluation by trypan blue staining.

Sensitivity to 5-FU. HMVEC or TSA cells were plated in 6-well dishes at a concentration of 2×10^5 cells/well. A20 or EL4 cells were cultured in 25-cm flasks (kept upright) at the same density. Twelve h later, increasing concentrations of 5-FU were added to the culture media. After 96 h, cell survival was assessed by trypan blue counting and spectrophotometric measurements at 490 nm. IC_{50} level was defined as the concentration of 5-FU that induced 50% cell killing compared with control untreated cells (27).

Experimental Tumor Models. All *in vivo* experiments were performed in accordance with the University of Miami animal care guidelines. BALB/c mice (8-week-old) were purchased from The Jackson Laboratory. A20 (10^6) or TSA (10^5) cells (in 100 μ l of PBS) were injected in the right flank of each mouse. Once tumors were established (4–5-mm diameter), mice were randomized in groups of five and six mice each. One group of mice received five daily i.p. injections of 30 mg/kg 5-FU. The other groups of mice received two injections, 3 days apart, of 2×10^7 of either HI-VSV, rVSV-GFP, rVSV-TK (two groups), or rVSV-C:U (two groups). Starting 24 h after the first virus injection, one group of mice that received an injection with rVSV-TK received 100 mg/kg GCV, daily, i.p. for 7 days, and one group of mice that received an injection with rVSV-C:U received 500 mg/kg 5-FC, daily, i.p. for 10 consecutive days. Tumors were measured every other day using a caliper, and tumor volumes were estimated using the following formula: volume = length \times width²/2. Mice were sacrificed when tumors measured >15 mm in any diameter. Results were reported as (a) mean tumor volume of each group at the time point when the first mouse had to be culled and (b) survival of each group of mice over the period of 45 days of follow-up. For statistical analysis, Student's *t* test was used (3). For rechallenge experiments, at the end of 45 days, surviving mice free of A20 cells, a group of five control mice, and the mice that previously received the injection with A20 cells but did not develop tumors were rechallenged with the same number of 10^5 A20 cells in the contralateral flank.

For 5-FU measurements, TSA tumors were established by s.c. injection of 10^5 TSA cells in the right flank of each mouse. When the tumors reached approximately 10 mm in diameter, mice were divided into two groups. One group received 90 mg/kg 5-FU i.p., and animals were sacrificed at different time points after the injection. Blood samples and tumors were collected. The other group of mice received one intratumoral injection of 2×10^7 rVSV-C:U followed 24 h later by 500 mg/kg 5-FC i.p. Animals were sacrificed at different time points after having received the i.p. injection, and blood samples and tumors were collected. The 5-FU extraction procedures were performed as described previously (16). Plasma was separated by centrifugation from blood collected via the retroorbital sinus in heparinized tubes. Fifty μ l of plasma were quenched with 500 μ l of ethyl acetate/2-propanol/0.5 M acetic acid (84:15:1) and stored at -20°C . At the time of extraction, the samples were thawed, vortexed, and centrifuged. The organic supernatant was evaporated in a Speed Vacuum Concentrator (Savant) and resuspended in 50 μ l of mobile phase, vortexed, sonicated for 10 s, centrifuged to remove particles, and analyzed by HPLC. 5-FU and 5-FC were separated isocratically on a Beckman Coulter Gold HPLC System equipped with 32 Karat software and photo diode array 168 detector. The eluates were monitored at 254 and 290 nm. A Whatman Partisil 5 SAX (4.6 \times 250-mm) column with matching guard cartridge was used at a flow rate of 0.5 ml/min. The mobile phase was 10 mM $\text{NH}_4\text{H}_2\text{PO}_4$ buffer (pH 3.8) containing 7% methanol. 5-FU and 5-FC eluted at 6.5 and 7.1 min, respectively.

Viral Titer Measurement in the Tumors. TSA tumors were established in BALB/c mice by s.c. infection of 10^5 TSA cells in the right flank. When tumors reached 10 mm in diameter, mice were randomized in three groups and treated intratumorally with two administrations, 3 days apart, of 2×10^7 HI-VSV (one group) or rVSV-C:U (two groups). One group of mice that received live virus intratumorally were also treated with 500 mg/kg 5-FC i.p. daily. Mice were sacrificed at days 2, 4, and 6, and the tumors were collected, weighed, and frozen. At the time of viral titer measurements, tumors were

thawed and homogenized in 1 ml of PBS, and virus was measured by standard plaque assay.

ELISPOT Assay. We followed the experimental protocol described previously in detail (28). Nunc MaxiSorp plates were coated with 100 μ l/well of a 0.5 μ g/ml solution of antineuronal IFN- γ antibodies and incubated overnight at room temperature. Spleens were collected from the three groups of two mice each (treated with HI-VSV, rVSV-C:U, or rVSV-C:U + 5-FC) sacrificed on day 6 of the above-described experiment (see "Viral Titer Measurement in the Tumors"). Spleen cells were suspended in 10% RPMI 1640 and plated in the coated wells in triplicate using four different cell concentrations in 2-fold dilutions (from 10^7 cells/well of the highest concentration). Spleen cells were cultured in the presence or absence of 10^5 irradiated TSA cells/well. The plates were incubated for 24 h at 37°C in 5% CO_2 . The plates were then washed and covered with biotinylated anti-IFN- γ monoclonal antibodies (100 μ l/well) and incubated for 90 min at room temperature. After additional washes, 100 μ l/well of 0.2 μ g/ml of streptavidin-alkaline phosphatase were added, and the wells were incubated for 60 min at room temperature. After washing again, 100 μ l of a 1:4 mixture of 3% melted low EEO type 1 agarose and 2.3 mM 5-bromo-4-chloro-3-indolyl phosphate in AMP buffer was added to each well. The developed spots were counted under the dissecting microscope. The triplicate wells with >50 and <100 spots were used to calculate the average of the two experiments \pm SD.

RESULTS

Generation and Characterization of VSV-Expressing CD4/UPRT. To evaluate whether VSV could be successfully generated to carry and express the *E. coli* CD4/UPRT (C:U) fusion gene, cDNA-encoding C:U was cloned into the pVSV-XN2 plasmid (pVSV-XN2-C:U), which carries the full-length antigenomic VSV DNA (21, 22). The additional foreign transcription unit (C:U) was inserted between the VSV glycoprotein (G) and the polymerase gene (L) in a position flanked by unique *Xho*I and *Nhe*I restriction sites (Fig. 1A). After transfection with pVSV-XN2-C:U, recombinant virus was recovered in BHK cells 48 h later, and plaque purification of resultant recombinant viruses (rVSV-C:U) was carried out as described previously (29).

To investigate whether the insertion of the foreign suicide gene affected viral replication and infectivity, we analyzed rVSV-C:U production per cell after infection of BHK cells at a MOI of 10. Accordingly, one-step growth curve studies indicated that rVSV-C:U exhibited similar growth characteristics to WT-VSV or rVSV expressing the GFP (rVSV-GFP) or TK (rVSV-TK) and reached 10^9 viruses/ml within 10 h (Fig. 1B). Indeed, rVSV-C:U induced almost complete cytolysis of BHK cells (MOI = 0.01) within 48 h, similar to WT VSV (Indiana strain), rVSV expressing TK, or rVSV expressing GFP (Fig. 1C). Because of the rapid viral-induced killing, the addition of 5-FC had very little additional effect on the kinetics of BHK cell death (data not shown). The levels of viral production were also similar for rVSV-C:U and other control VSVs, even after infection of BHK cells with very low doses of virus (Fig. 1D). In conclusion, the C:U fusion gene does not appear to affect VSV replication, and rVSV-C:U exhibits *in vitro* growth characteristics comparable with WT VSV after infection of BHK cells.

VSV Expresses High Levels of Functional C:U. Confirmation of expression of the C:U gene by rVSV-C:U was next examined by measuring the enzymatic activity of CD using spectrophotometric assay and chromatographic enzyme assays as described previously (15). Essentially, for chromatographic identification, BHK cells were infected with rVSV-C:U at MOI = 0.1 or 5.0, and cell lysates were retrieved at various times postinfection. This analysis indicated that labeled cytosine was effectively metabolized to uracil in lysates from rVSV-C:U-infected cells but not rVSV-GFP-infected cells (Fig. 2A). Spectrophotometric analysis confirmed expression and indicated that as much as 70 nm 5-FU/min was produced using cell lysates prepared

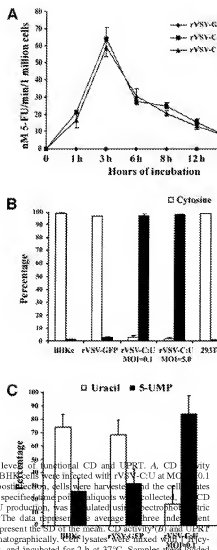


Fig. 2. rVSV expresses high level of functional CD and UPR. A, CD activity measured spectrophotometrically. BHK cells were infected with rVSV-C:U at MOI of 0.1 or 5.0 or with rVSV-GFP. Six h postinfection, cells were harvested and the cells were incubated with 5-FC. At the specified time points, aliquots were collected. CD activity, expressed as rate of 5-FU production, was assayed using spectrophotometric measurements at A_{390} and A_{235} . The data represent the average of three independent experiments, and the error bars represent the SD of the mean. CD activity (B) and UPR activity (C) were measured chromatographically. BHK lysates were mixed with deuterio-cytosine and [3 H]uracil, respectively, and incubated for 2 h at 37°C. Samples were loaded on TLCs. Bands corresponding to cytosine and uracil were evaluated by liquid scintillation counting, and results are presented as a percentage of total scintillation count for each sample. 293T cells transiently transfected with an empty vector or with the *E. coli* C:U fusion gene were used as control for the CD activity experiment. The data represent the average of two or three independent experiments, and the error bars represent the SE.

Our data demonstrated that infection of TSA cells with high titers of rVSV-GFP (MOI = 10) was not influenced by pretreatment with 10 μ M 5-FU or 10 mM 5-FC used in the assay (the cells' IC_{50} for 5-FU was determined at 3 μ M). The percentage of infected cells was 80–90% by 4 h (Fig. 3D). At lower dose infection (MOI = 0.1), the percentage of infected cells at 12 h was slightly lower after 5-FU pretreatment (Fig. 3D), although by 24 h almost 100% of cells were infected (data not shown). Viral production in TSA cells pretreated with 5-FU was <1 log lower than in control cells, remaining at high levels of 2×10^8 . Cell death evaluation by trypan blue exclusion showed almost complete cell death within 24 h, both in control and in 5-FC- and 5-FU-pretreated conditions (data not shown). Taken together, these *in vitro* assays demonstrate that exposure to relatively

high levels of 5-FC and 5-FU did not significantly influence VSV replication and oncolytic activity.

Evaluation of the *In Vitro* Oncolytic Efficiency of rVSV-C:U/5-FC. We next evaluated the combined oncolytic efficiency of rVSV-C:U/5-FC on different cancer cell lines or normal cells. Accordingly, rVSV-C:U, rVSV-GFP, HI-VSV, or WT-VSV was used to infect TSA, K562, 8226/Dox40, and EL4 cancer cells or normal HMVEC cells at a MOI of 0.1. Results indicated that all recombinant viruses killed the cancer cell lines ($>75\%$ cell death) within 24 h (Fig. 4A). In contrast, rVSV-C:U + 5-FC or VSV-GFP was found to induce a low fraction of HMVEC killing (20–25%) when treated similarly (Fig. 4A). Pretreatment with type I IFN completely prevented virus replication in the normal HMVEC cells but not in the cancer cell lines. For example, rVSV-C:U titers were 2000 times lower in infected HMVEC cells than in 8226/Dox40 cells by 24 h postinfection and decreased to an undetectable level in HMVEC cells, but not 8226/Dox40 cells, when pretreated with IFN- α (Fig. 4, B and C). These data confirmed earlier findings that at least one potential mechanism of VSV-mediated oncolysis exploits defects in the IFN pathway present in tumor cells (30). In addition, these data demonstrate the selective oncolytic efficiency mediated by rVSV-C:U/5-FC.

Because rVSV-C:U infection in the absence of 5-FC clearly exhibits a viral-induced oncolytic effect alone, we next examined the contribution of 5-FU in cancer cell killing. For this experiment, mouse A20 lymphoma cells were used because they were found to exhibit different levels of virus-induced CPE when infected with rVSV-GFP and observed by fluorescent microscopy. For reasons that remain to be clarified, a small population of A20 cells was found to remain uninfected, possibly because some cells differentiated into a subpopulation that did not interact efficiently with the virus (Fig. 4D). However, in general, a high level of viral replication was achieved in the infected population, with viral titers reaching 10^7 by 24 h postinfection (data not shown). Nevertheless, when 5-FC (1 mM) was added to the culture medium harboring A20 cells infected with rVSV-C:U, almost complete cell death could be demonstrated by trypan blue staining after 48 h (Fig. 4E). In contrast, the dose of 5-FC used (1 mM) did not affect A20 cell growth when used alone. To further distinguish between the contribution of the virus alone and 5-FU bioactivation in A20 cell killing, cells were infected with rVSV-C:U, rVSV-GFP, or HI-VSV, and after 6 h, they were mixed at different ratios with uninfected cells and cultured in the presence of 1 mM 5-FC. Cell viability was measured after 48 h. The difference observed between HI-VSV + 5-FC and rVSV-GFP + 5-FC essentially represents the VSV effect of the virus alone, whereas the difference between rVSV-GFP + 5-FC and rVSV-C:U + 5-FC accounts for the additional oncolytic effect generated by 5-FU (Fig. 4F). Collectively, these data indicate increased killing of A20 cells using rVSV-C:U + 5-FU, emphasizing that bystander effects can contribute toward the efficacy of oncolysis.

***In Vivo* Activity of the rVSV-C:U/5-FC System.** To evaluate the oncolytic effect of the rVSV-C:U system *in vivo*, we chose to investigate two aggressive s.c. tumor models, A20 and TSA, that differed slightly in their response to 5-FU. For example, the A20 lymphoma model showed a higher sensitivity to 5-FU (IC_{50} = 1 μ M) compared with the TSA mammary prototype (IC_{50} = 3–4 μ M). Our goals were to evaluate the efficiency of the VSV-directed C:U/5-FC model and to compare it with the previously published rVSV-TK/GCV model. Accordingly, s.c. tumors were grown in the left flank of BALB/c immunocompetent mice. When tumors became palpable, HI-VSV, rVSV-C:U, or rVSV-TK was injected intratumorally (2×10^7 plaque-forming units), followed or not followed by the administration of the respective prodrug, 5-FC or GCV. As an additional control, 5-FU was administered daily for 5 days i.p. Virus treatments were repeated once

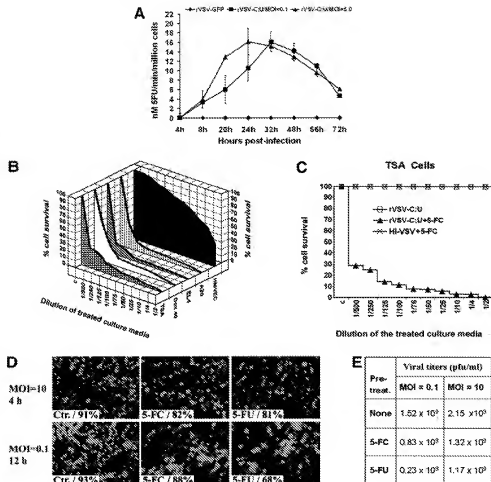


Fig. 3. 5-FU generated by the rVSV-C:U-prodrug system produces an efficient bystander effect and does not interfere significantly with the viral replication and oncolysis. **A**, BHK cells were infected with rVSV-C:U at MOI = 0.1 or 5.0 or with rVSV-GFP and treated with 5-FU. At the specified time points, aliquots of the conditioning media were collected, and the rate of 5-FU generated in the media was determined using spectrophotometric measurements at A_{250} and A_{280} . **B** and **C**, evaluation of the bystander effect. HI-VSV, A20, EL4, 8226/Dox40, or TSA cells were infected with rVSV-C:U at MOI = 0.1 or mock infected (HI-VSV) and treated or not treated with 5-FU. Culture media aliquots collected at 24 h were treated at 60°C for 10 min, to inactivate the virus and mixed in different ratios with fresh corresponding media before being added to freshly cultured cells. Cell survival was evaluated by trypan blue exclusion after 96 h. **D** and **E**, 5-FU and 5-FU do not interfere significantly with viral replication and oncolysis. TSA cells were pretreated with 10 mM 5-FU or 10 μ M 5-FU for 24 h. Pretreated as well as untreated control cells were infected with rVSV-GFP at MOI = 0.1 or 10 and covered with fresh media. At 4 and 12 h, cells were analyzed and photographed by fluorescent microscope. Culture media samples were collected at 24 h, and viral titers were measured by standard plaque assay.

more after 3 days, and tumor volumes were monitored every other day. For these experiments, two end points were established. First, survival was monitored with the specification that mice were sacrificed when the largest tumor diameter reached 15 mm. Second, evaluation of the mean tumor volumes was determined at a time when the first animal in the experiment was sacrificed.

This study indicated that there was a significant reduction in A20 or TSA tumor growth using either rVSV-TK or rVSV-C:U in the absence of prodrug, compared with HI-VSV ($P < 0.001$; Fig. 5A for A20 and Fig. 6A for TSA). However, even more potent tumor inhibition was found in the virus suicide gene/prodrug groups (rVSV-TK/GCV and rVSV-C:U/5-FU; Figs. 5A and 6A). Student's *t* test analysis indicated that animals harboring A20 or TSA and receiving rVSV-C:U/5-FU treatment fared better than those receiving rVSV-C:U alone ($P < 0.05$). The same analysis for rVSV-TK/GCV did not reach statistical significance, indicating that generation of 5-FU may be a better prodrug system in these tumor models. Statistical significance was seen between the rVSV-C:U + 5-FU group and 5-FU group [$P < 0.001$ in the TSA experiments (Figs. 5A and 6A) and $P < 0.05$ in the A20 experiment]. There was no difference between the HI-VSV and the HI-VSV + 5-FU groups in either of the experiments (data not shown). Indeed, although many of the tumors appeared of similar size in a number of differently treated groups after 14 days, tumors in all groups except for the rVSV-C:U + 5-FU-treated group grew rapidly. Indeed, animals with A20 tumors survived for the duration of the experiment (45 days) when treated with rVSV-C:U + 5-FU (Fig. 5B). In the control groups HI-VSV or HI-VSV + 5-FU, all animals had to be sacrificed after 20 days. By 90 days, four of six animals in the

rVSV-C:U + 5-FU group, two of five animals in the rVSV-TK + GCV group, one of five animals in the rVSV-C:U group, and one of five animals in the 5-FU group remained alive and free of tumors. In the TSA model, the same statistically significant difference in survival was observed between the rVSV suicide gene + prodrug groups and the control groups ($P < 0.05$; data not shown). The combined intratumoral administration of both rVSV-TK and rVSV-C:U (in conjunction with GCV and 5-FU) did not significantly achieve better results than rVSV-C:U + 5-FU in the A20 model alone (data not shown). Collectively, our data indicate that because of increased bystander effect, rVSV-C:U may be a better oncolytic agent than rVSV-TK.

The Induction of Antitumor Immune Responses by rVSV-C:U. Previous data have demonstrated that lysis of cells, e.g., through virus replication (CPE), can result in the enhanced generation of CTL activity, arguably by facilitating tumor antigen uptake by professional antigen-presenting cells (31, 32). To evaluate this, animals exhibiting no tumor formation after 45 days in the previously presented A20 model were rechallenged with the same number of A20 cells injected in the opposite flank of the animal (six of six animals in the rVSV-C:U + 5-FU group, two of five animals in the rVSV-C:U group, three of five animals in the rVSV-TK + GCV group, one of five animals in the 5-FU group, and zero of five animals in HI-VSV, HI-VSV + 5-FU, and rVSV-TK groups). Significantly, none of the rechallenged animals developed tumors at the site of cancer cell readministration, in contrast with a control group of new animals not treated previously, 4 of 5 of which developed tumors within 15 days postimplantation. These data indicate activation of the immune system against A20

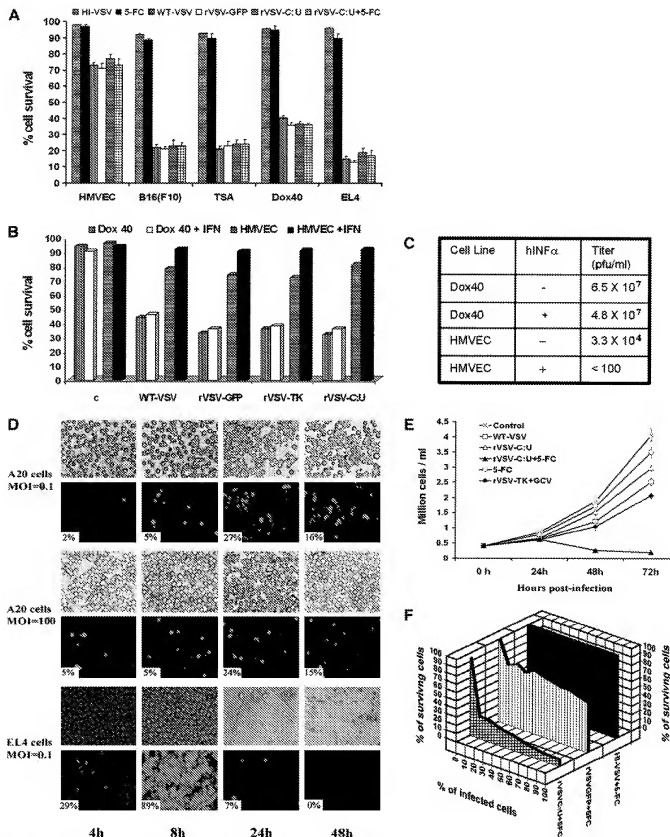


Fig. 4. Virus/suicide gene system retains selective oncolytic efficiency. A, tumor cells are sensitive to rVSV-C:U/5-FC. HMVEC, TSA, B16(F10), 8226/Dox40, or EL4 cells were infected with rVSV-C:U at MOI = 0.1, in the presence or absence of 5-FC added to the culture media. Cells treated with 5-FC alone, HI-VSV, or WT-VSV at MOI = 0.1 were used as control. Cell survival was evaluated by trypan blue exclusion after 18 h. B and C, Dox40 cells are resistant to IFN antiviral action. HMVEC or 8226/Dox40 cells were pretreated with 1000 IU/ml hINF α . After 24 h, pretreated as well as untreated control HMVEC and 8226/Dox40 cells were infected with WT-VSV, rVSV-GFP, rVSV-TK, or rVSV-C:U at MOI = 0.1. Twenty-four h postinfection, samples of culture media were collected for viral titer measurement, and cell viability was determined by trypan blue exclusion. D, A20 cells are less sensitive to infection with VSV. A20 cells or control EL4 cells were infected with rVSV-GFP at MOI = 0.1 or 100. At 4, 8, 24, and 48 h postinfection, cells were resuspended,

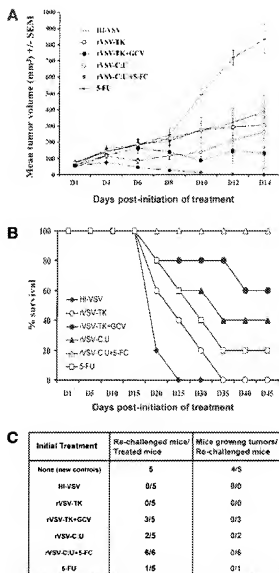


Fig. 5. RSVSV expressing C-U inhibits the tumor growth of the syngeneic A20 B-cell lymphoma tumor model in immunocompetent BALB/c mice. A, 10^6 A20 cells were injected s.c. in the flank of immunocompetent BALB/c mice. When tumors became palpable, mice were treated with 5FU i.p., 2×10^6 plaque forming units of rVSV-TK, rVSV-TK, or rVSV-C:U injected intratumorally twice a day at 3-day intervals. Mice that received the active virus were randomized to receive or not receive GCV i.p. (for rVSV-TK) or 5-FU i.p. (for rVSV-C:U). Tumor size was measured every other day. Tumor volumes were calculated and are presented as average \pm SE. Mice were sacrificed when the tumors reached 15 mm in the largest diameter. B, survival of the different therapeutic groups is presented over a period of follow-up of 45 days. C, table showing survival of animals from original experiments that were re-challenged with A20 cells.

cells, which may have contributed toward the antitumor activity of rVSV-C:U/5-FC.

To further characterize the mechanisms of rVSV-C:U/5-FC action, we measured the activation of T cells in the spleens of mice receiving tumor therapy by performing ELISPOT assay. Mice with s.c. TSA tumors were treated with HI-VSV, rVSV-C:U, or rVSV-C:U + 5-FC.

Six days after the initiation of treatment, mice were sacrificed, and spleens were collected. Mononuclear cells were isolated, and IFN- γ release by single cells was measured in the presence or absence of irradiated TSA cells as a source of *in vitro* stimulating antigen. We found that there were 30 and 44 times more T cells activated and secreting IFN- γ in the spleens of the mice that were treated with rVSV-C:U and rVSV-C:U + 5-FC, respectively, than in the spleens of mice bearing TSA tumors that received control HI-VSV (Fig. 6B). The number of secretory cells doubled in both viral treatment conditions when they were exposed to TSA cells as a source of antigen. Interestingly, the number of activated T cells was found to be almost double when rVSV-C:U was combined with generated 5-FU. These data indicate a strong immune component in the antitumor efficiency of rVSV-C:U/5-FC therapy.

We next evaluated, by HPLC, 5-FU levels in the blood after one single maximally tolerated dose of 5-FU or after intratumoral administration of rVSV-C:U in the presence of systemic 5-FC. Blood level measurement in samples collected at different time points revealed that systemic 5-FU administration produced high early peaks (up to 4.4 times higher than the rVSV-C:U + 5-FC by 15 min; Fig. 6C). However, exogenously added levels drop immediately, *in vivo*, so that after 25 min, 5-FU produced by the viral/prodrug treatment was detected at higher concentrations and maintained therapeutic levels over the next hour, thus indicating longevity of the system. We could not find any difference in the viral titers achieved by the rVSV-C:U virus in the presence or absence of prodrug administration when measured on day 4 (already 2 days through 5-FC treatment) or day 6 (after four administrations of 5-FC), suggesting that 5-FC and 5-FU did not affect VSV propagation in the tumor (Fig. 6D).

Thus, we demonstrate the presence of potent viral, chemotherapeutic, and antitumor immune components after treatment with rVSV-C:U/5-FC, indicating that this system may have promise for development as a therapeutic against malignant disease.

DISCUSSION

VSV has been recently characterized as a selective oncolytic virus showing significant *in vitro* and *in vivo* potential as a novel therapy for cancer (30). Previous studies have demonstrated that VSV is capable of inhibiting the growth of tumors harboring common genetic abnormalities such as p53, *myc*, or *ras* aberrations, both after local intratumoral administration and after systemic i.v. treatment (33). Furthermore, VSV has been shown to eradicate tumors in immunocompetent hosts without any substantial virus-associated CPE occurring in normal tissue (33). Recombinant adaptations of the virus that contain antitumor genes afford the opportunity to further increase the oncolytic efficiency of VSV, with concomitant specificity and attenuation (3). In this regard, previous studies have shown that the expression of cytokines such as interleukin-4 and suicide genes such as herpes simplex virus-TK increased the ability of VSV to suppress tumor growth, compared with WT-VSV, in melanoma and mammary cell carcinoma models (3).

A potential problem concerning viral tumor therapy in immunocompetent hosts remains the response of the immune system to virus infection, which may suppress a required oncolytic effect through inhibition of virus spread (35). One strategy to help avoid this di-

and samples were analyzed by fluorescent microscopy and flow cytometry to calculate the percentage of GFP-expressing cells. E, rVSV-C:U/5-FC displays potent oncolytic activity against A20 cells. A20 cells were infected with rVSV-C:U at MOI = 0.1, in the presence or absence of treatment with the prodrug 5-FC. A20 cells treated with 5-FC alone, infected with rVSV-TK in the presence of GCV, or infected with WT VSV at MOI = 0.1 served as controls. Number of living cells was counted after trypan blue staining at 24, 48, and 72 h. F, measurement of 5-FU by HPLC after intratumoral effect in A20 lymphoma cells. A20 cells were infected with HI-VSV, rVSV-GFP, or rVSV-C:U at MOI = 0.1. After 4 h, infected cells were mixed in different proportions with noninfected cells, and 5-FC was added to the conditioning media. Cell survival was evaluated by trypan blue staining after 48 h. The data presented correspond to the average of three independent experiments.

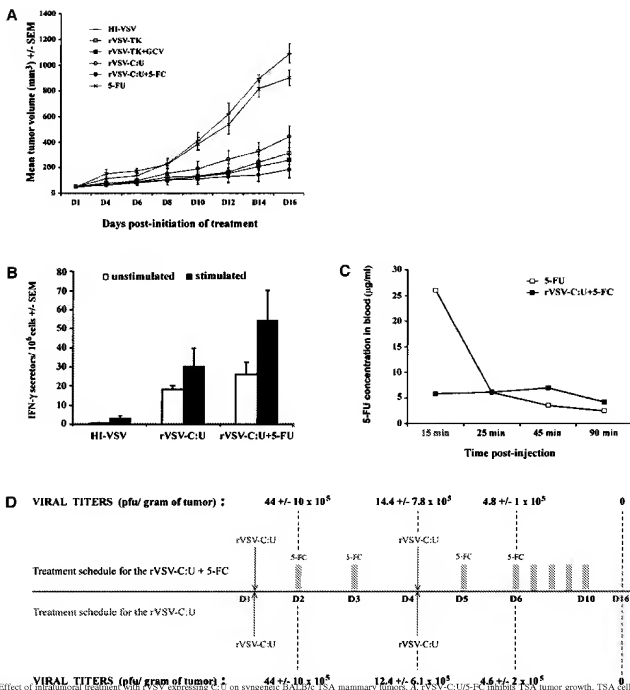


Fig. 6. Effect of intratumoral treatment with rVSV expressing C on syngeneic BALB/c TSA mammary tumors. A, rVSV-C:U/5-FU inhibit TSA tumor growth. TSA cells (10^5) were injected s.c. in BALB/c immunocompetent mice. When tumors became palpable, mice were treated with 5-FU i.p. or received 2×10^7 HI-VSV, rVSV-TK, or rVSV-C:U intratumorally, two administrations at 3-day intervals. Mice treated with the active virus received or did not receive GCV (for rVSV-TK) or 5-FU (for rVSV-C:U). Mean tumor volume \pm SE is presented for all treatment groups. B, CTL activation after rVSV-C:U/5-FU treatment. BALB/c mice with TSA tumors grown s.c. received HI-VSV or rVSV-C:U as two intratumoral administrations, 3 days apart, with or without concomitant systemic administration of 5-FU. At day 6 postinitiation of treatment, mice were sacrificed, and the spleens were used in the ELISPOT assay. The number of activated splenocytes that secrete IFN- γ , counted in triplicate in two independent experiments, is presented as the mean \pm SE. C, *in vivo* 5-FU levels in treated animals. In animals carrying s.c. TSA tumors, blood samples were drawn at different time points after systemic administration of a single maximally tolerated dose of 5-FU or after one systemic administration of 5-FU after intratumoral administration of rVSV-C:U. 5-FU level analysis was performed by HPLC. D, intratumoral viral replication is not affected by the suicide gene/prodrug system. Viral titers were measured by standard plaque assay at different time points in the TSA tumors grown s.c. and treated with rVSV-C:U intratumorally, in the presence or absence of the systemic 5-FU administration.

lemma may be the addition of a system capable of exerting a strong bystander effect, which may enhance the oncolytic efficacy of the virus therapy by eliminating uninfected tumor cells. In contrast to the TK/GCV system, the CDUPRT/5-FU system may confer a stronger bystander activity that is independent of intercellular junctions (36–38). This may be important in the treatment of tumors with down-

regulated intercellular gap junctions (36). Previous attempts to use viral agents in the therapy of cancer have been limited by lack of selectivity, inefficient replication of the virus in the tumor, or apprehension associated with the transforming potential of the virus itself (2, 39). Given this, we therefore decided to evaluate the antitumor/bystander potential of the recently characterized *E. coli* CDUPRT

fusion suicide gene with the actively replicative, selectively oncolytic rVSV as a viral vector (15, 17).

The generation of rVSV-C:U was achieved, and impressively high levels of CD and UPRT activity were apparent. Spectrophotometric measurements of CD activity up to 64 ± 7 nm 5-FU/min were produced by approximately 1 million infected BHK cells. This is in comparison with a recently published value of 10 ± 2 nm 5-FU/min produced by 1 million BNL1 hepatocellular carcinoma cells that were retrovirally transduced with the *E. coli* CD gene (24). The CD gene from *E. coli* also compares favorably with results reported previously using the yeast CD/UPRT fusion gene (15). In addition, our results indicate that rVSV-C:U + 5-FC generates a very strong bystander killing effect. The amount of 5-FU produced by recombinant viruses in the media essentially eradicated >90% of a number of tumor cell types tested. This observation indicates that 5-FU effectively diffuses in and of cells and, unlike TK/GCV system, does not require cell-to-cell contact for bystander activity (36).

Other potential issues regarding virus gene therapy include taking into consideration the possibility that the rapid oncolytic effect of the virus itself could limit the amount of chemotherapeutic agent produced in the cell. Conversely, the chemotherapeutic drug could conceivably limit viral replication and subsequent propagation in the tumor (40, 41). However, we demonstrated high levels of 5-FU production in cancer cells *in vitro* and intratumorally *in vivo*. *In vitro* we showed that CD continues to transform the provided prodrg up to 72 h postinfection. In addition, our *in vitro* and *in vivo* data indicate that the generated 5-FU and the administered 5-FC do not significantly inhibit VSV replication, and the viral titers achieved in the tumors by the rVSV-C:U were similar in the presence or absence of prodrg administration. A probable explanation is that VSV is a RNA virus, and its cytoplasmic replication is less affected by the multiple mechanisms by which 5-FU inhibits DNA and RNA synthesis, altering cell growth and survival. This demonstration makes the rVSV a specifically suitable viral vector for the CD/UPRT/5-FC suicide gene therapy.

We have recently indicated that VSV selectively replicates in tumor cells because of such cells harboring a flawed IFN system. In this regard, IFN pretreatment completely protected the normal cells against rVSV-C:U replication and cytotoxicity, as demonstrated previously for the VSV oncolytic agents (30). In contrast, VSV oncolysis of tumor cells was extremely prompt and efficient, masking the contribution of the suicide gene system in all cancer cell lines except the mouse A20 B-cell lymphoma cells. In this particular cancer cell model, rVSV-C:U induced almost complete cytotoxicity only in the presence of the prodrg 5-FC, demonstrating a synergistic effect of the rVSV and the CD/UPRT/5-FC components in oncolysis of the lymphoma cells. This may reflect that some lymphoma cell types, such as chronic lymphocytic leukemia, exhibit selective resistance to VSV infection, perhaps because of tropism issues. Nevertheless, this finding allowed the opportunity to evaluate the bystander effect of the rVSV-C:U system. Accordingly, rVSV-C:U exhibited greater therapeutic oncolytic activity in the presence of systemically administered 5-FC compared with rVSV-C:U virus alone and with the previously published rVSV-TK/GCV system. Similar results were observed in the TSA mammary tumor model. The contribution of the viral oncolysis to suppression of the tumor can be compared with the measured effect of rVSV-C:U alone, in the context of demonstrating similar viral titers in tumors injected with the virus and exposed or not exposed to systemic 5-FC. The role of the generated 5-FU is revealed by the statistically greater therapeutic efficiency of rVSV-C:U + 5-FC compared with rVSV-C:U alone in both tumor models.

Another potential antitumor action invoked by rVSV-C:U/5-FC is the stimulation of the immune system. Robust immune responses have

been documented previously in experiments using CD-based therapies, as demonstrated by the presence of intense inflammatory infiltrates found within the treated tumors (42), production of cytokines (43, 44), eradication of distant tumors (45), reduction of the bystander effect in nude mice (24), or after sublethal irradiation (46, 47). Although virus replication itself induces strong CTL responses against viral antigens presented on the surface of infected cells, it is plausible that CTL responses may be invoked against tumor antigens by this treatment also (48, 49). In our analysis, the generation of CTLs after VSV suicide gene treatment was indicated by the lack of any tumor growth in A20 cancer model rechallenged experiments. In addition, ELISPOT analysis confirmed the presence of IFN- γ -producing T cells specific to the tumor only in animals receiving rVSV-C:U treatment.

Collectively, our data indicate that VSV is able to generate high levels of CD and UPRT. In addition, 5-FU was found to participate in the killing of infected tumor cells and to exhibit bystander effect. Future studies could conceivably enhance VSV-based oncolytic activities by combining suicide genes with immunomodulatory cytokines or with radiotherapy, thus exploiting the radiosensitizing potential of 5-FU (50).

ACKNOWLEDGMENTS

We thank Heather Ezelle, Siddarth Balachandran, Dubravka Markovic, Mario Moleno, and Masatsugu Obuchi for technical assistance; Dr. L. H. Boise for the gift of 8826/Dox40 cells; and Dr. J. Rosenblatt for the gift of EL4 and A20 cell line.

REFERENCES

- Greco, O., and Dachs, G. U. Gene directed enzyme/prodrug therapy of cancer: historical appraisal and future perspectives. *J. Cell. Physiol.*, 187: 22–36, 2001.
- Martinez, R. L. Conditionally replicating herpes vectors for cancer therapy. *J. Clin. Invest.*, 104: 841–846, 2000.
- Fernandez, M., Ponsnick, M., Markovic, D., and Barber, G. N. Genetically engineered vesicular stomatitis virus in gene therapy: application for treatment of malignant disease. *J. Virol.*, 76: 895–904, 2002.
- Wagner, R. K., and Rose, J. K. Rhodoviridae: the viruses and their replication. *In: R. J. Whitley, B. N. Fields, D. M. Knipe, and P. M. Howley (eds.), Fields Virology*, 3rd ed., pp. 1121–1161. Philadelphia: Lippincott-Raven Publishers, 1996.
- Johnson, J. E., Schnell, M. J., Buonocore, L., and Rose, J. K. Specific targeting to CD4+ cells of recombinant vesicular stomatitis viruses encoding human immunodeficiency virus envelope proteins. *J. Virol.*, 71: 5060–5068, 1997.
- Reif, J. S., Webb, P. A., Monath, T. P., Emerson, J. K., Poland, J. D., Kemp, G. E., and Chola, G. Epizootic vesicular stomatitis virus in Colorado, 1982: infection in occupational risk groups. *Am. J. Trop. Med. Hyg.*, 36: 177–182, 1987.
- Barber, G. N. The interferons and cell death: guardians of the cell or accomplices of apoptosis? *Semin. Cancer Biol.*, 10: 103–111, 2000.
- Stojdl, D. F., Lichty, B., Knowles, S., Maris, R., Atkins, H., Sonenberg, N., and Bell, J. C. Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus. *Nat. Med.*, 6: 821–825, 2000.
- Moolten, F. L. Tumor chemosensitivity conferred by inserted herpes thymidine kinase genes: paradigm for a prospective cancer control strategy. *Cancer Res.*, 46: 5276–5281, 1986.
- Mullen, C. A., Kilstrup, M., and Blaese, R. M. Transfer of the bacterial gene for cytosine deaminase to mammalian cells confers lethal sensitivity to 5-fluorocytosine: a negative selection system. *Proc. Natl. Acad. Sci. USA*, 89: 33–37, 1992.
- Aghi, M., Krann, C. M., Chou, T. C., Breakefield, X. O., and Chioia, E. A. Synergistic anticancer effects of ganciclovir/thymidine kinase and 5-fluorocytosine/cytosine deaminase gene therapies. *J. Natl. Cancer Inst. (Bethesda)*, 90: 370–380, 1998.
- Huber, B. E. Gene therapy strategies for treating neoplastic disease. *Ann. N. Y. Acad. Sci.*, 716: 6–11, discussion 20–22, 1994.
- Trinh, Q. T., Austin, E. A., Murray, D. M., Knick, V. C., and Huber, B. E. Enzyme/prodrug gene therapy: comparison of cytosine deaminase/5-fluorocytosine versus thymidine kinase/ganciclovir enzyme/prodrug systems in a human colorectal carcinoma cell line. *Cancer Res.*, 55: 4808–4812, 1995.
- Kanai, F., Kawakami, T., Hamada, H., Sadata, A., Yoshida, Y., Tanaka, T., Ohashi, M., Tateishi, K., Shiratori, Y., and Onata, M. Adenovirus-mediated transduction of *Escherichia coli* aryl phosphoribosyltransferase gene sensitizes cancer cells to low concentrations of 5-fluorouracil. *Cancer Res.*, 58: 1946–1951, 1998.
- Erbe, P., Regulier, E., Kintz, J., Leroy, P., Potevin, Y., Exinger, F., Jond, R., and Melich, M. *In vivo* cancer gene therapy by adenovirus-mediated transfer of a bifunctional yeast cytosine deaminase/aryl phosphoribosyltransferase fusion gene. *Cancer Res.*, 60: 3813–3822, 2000.

16. Adachi, Y., Tamiya, T., Ichikawa, T., Terada, K., Ono, Y., Matsumoto, K., Furuta, T., Hamada, H., and Ohnoto, T. Experimental gene therapy for brain tumors using adenovirus-mediated transfer of cytosine deaminase gene and uracil phosphoribosyl-transferase gene with 5-fluorocytosine. *Hum. Gene Ther.*, **11**: 77–89, 2000.
17. Chung-Flye, G. A., Chen, M. J., Green, N. K., Burton, A., Anderson, D., Maitner, V., Searle, P. F., and Kerr, D. J. *In vivo* gene therapy for colon cancer using adenovirus-mediated, transfer of the fusion gene cytosine deaminase and uracil phosphoribosyl-transferase. *Gene Ther.*, **8**: 1547–1554, 2001.
18. Grad, J. M., Bahls, N. J., Reis, L., Oshiro, M. M., Dalton, W. S., and Boise, L. H. Ascorbic acid enhances arsenite trioxide-induced cytotoxicity in multiple myeloma cells. *Blood*, **98**: 895–913, 2001.
19. Kunitz, H., Fedoroff, H. J., Challa-Eld, P. M., Halterman, M., Day, B., Atkinson, M., Panielles, V., and Rosenblatt, J. D. Eradication of pre-established lymphoma using herpes simplex virus amplicon vectors. *Blood*, **93**: 643–654, 1999.
20. Tolba, K. A., Bowers, W. J., Muller, J., Houseknecht, V., Giuliano, R. E., Fedoroff, H. J., and Rosenblatt, J. D. Herpes simplex virus (HSV) amplicon-mediated co-delivery of secondary lymphoid tissue chemokine and CD40L results in augmented antitumor activity. *Cancer Res.*, **62**: 6545–6551, 2002.
21. Schnell, M. J., Buonocore, L., Whitt, M. A., and Rose, J. K. The minimal conserved transcription start signal promotes stable expression of a foreign gene in vesicular stomatitis virus. *J. Virol.*, **70**: 2318–2323, 1996.
22. Bortz, E., Gerlach, J., Johnson, J. E., and Rose, J. K. Replication-competent rhabdoviruses with human immunodeficiency virus type 1 coxals and green fluorescent protein: entry by a pH-independent pathway. *J. Virol.*, **73**: 6937–6945, 1999.
23. Lawson, N. D., Stillman, E. A., Whitt, M. A., and Rose, J. K. Recombinant vesicular stomatitis viruses from DNA. *Proc. Natl. Acad. Sci. USA*, **92**: 4477–4481, 1995.
24. Kunyama, S., Kikukawa, M., Masui, K., Okuda, H., Nakatani, T., Sakamoto, T., Yoshiji, H., Fukui, H., Ikenaka, K., Mullen, C. A., and Tsuji, T. Cytosine deaminase/5-fluorocytosine gene therapy can induce efficient anti-tumor effects and protective immunity in immunocompetent mice but not in athymic nude mice. *Int. J. Cancer*, **81**: 592–597, 1999.
25. Wang, Z. H., Zagzag, D., Zeng, B., and Kolodny, E. H. *In vivo* and *in vitro* glioma cell killing induced by an adenovirus expressing both cytosine deaminase and thymidine kinase and its association with interferon- γ . *J. Neuropathol. Exp. Neurol.*, **58**: 847–858, 1999.
26. Nakamura, H., Mullen, J. T., Chandrasekar, S., Pawlik, T. M., Yoon, S. S., and Tanabe, K. Multimodality therapy with a replication-conditional herpes simplex virus 1 mutant that expresses yeast cytosine deaminase for intratumoral conversion of 5-fluorocytosine to 5-fluorouracil. *Cancer Res.*, **61**: 5447–5452, 2001.
27. Miller, C. R., Williams, C. R., Buchsbaum, D. J., and Gillespie, G. Y. Intratumoral 5-fluorouracil produced by cytosine deaminase/5-fluorocytosine gene therapy is effective for experimental human glioblastomas. *Cancer Res.*, **62**: 773–780, 2002.
28. Adkins, B., Bu, Y., Cepero, E., and Perez, R. Exclusive Th2 primary effector function in spleens but mixed Th1/Th2 function in lymph nodes of murine neonates. *J. Immunol.*, **164**: 2347–2353, 2000.
29. Schnell, M. J., Buonocore, L., Kretzschmar, E., Johnson, E., and Rose, J. K. Foreign glycoproteins expressed from recombinant vesicular stomatitis viruses are incorporated efficiently into virus particles. *Proc. Natl. Acad. Sci. USA*, **93**: 11359–11365, 1996.
30. Balachandran, S., and Barber, G. N. Vesicular stomatitis virus (VSV) therapy of tumors. *UTMB Life*, **50**: 135–138, 2000.
31. Belfinger, C., Fulda, S., Kammertons, T., Meyer, E., Uckert, W., and Debatin, K. M. Herpes simplex virus thymidine kinase/ganciclovir-induced apoptosis involves ligand-independent death receptor aggregation and activation of caspases. *Proc. Natl. Acad. Sci. USA*, **96**: 8699–8704, 1999.
32. Berwin, B., Reed, R. C., and Nichevita, C. V. Virally induced lytic cell death elicits the release of immunogenic GRP94/gp96. *J. Biol. Chem.*, **276**: 21083–21088, 2001.
33. Balachandran, S., Porosnicu, M., and Barber, G. N. Oncolytic activity of vesicular stomatitis virus is effective against tumors exhibiting aberrant p53, Ras, or myc function and involves the induction of apoptosis. *J. Virol.*, **75**: 3474–3479, 2001.
34. Rose, N. F., Marx, P. A., Luckay, A., Nixon, D. F., Moretto, W. J., Donahoe, S. M., Monteleoni, D., Roberts, A., Buonocore, L., and Rose, J. K. An effective AIDS vaccine based on live attenuated vesicular stomatitis virus recombinants. *Cell*, **106**: 539–549, 2001.
35. Kim, D., Martuza, R. L., and Zwiebel, J. Replication-selective virotherapy for cancer: biological principles, risk management and future directions. *Nat. Med.*, **7**: 781–787, 2001.
36. Mesnil, M., Piccoli, C., Tiraby, G., Willecke, K., and Yamasaki, H. Bystander killing of cancer cells by herpes simplex virus thymidine kinase gene is mediated by connexins. *Proc. Natl. Acad. Sci. USA*, **93**: 1831–1835, 1996.
37. Morris, J. C. Enzyme/prodrug-based tumor vaccination: all politics (and immunity) are local. *J. Natl. Cancer Inst.* (Bethesda), **91**: 1986–1989, 1999.
38. Lawrence, T. S., Rehentulla, A., Ng, E. Y., Wilson, M., Trosko, J. E., and Stetson, P. L. Preferential cytotoxicity of cells transduced with cytosine deaminase compared to bystander cells after treatment with 5-fluorocytosine. *Cancer Res.*, **58**: 2588–2593, 1998.
39. Hette, C., Hermiston, T., Johnson, L., Brooks, G., Sampson-Johnnes, A., Williams, A., Hawkins, L., and Kim, D. An adenovirus E1A mutant that demonstrates potent and selective systemic anti-tumoral efficacy. *Nat. Med.*, **6**: 1134–1139, 2000.
40. McCart, J. A., Puhlmann, M., Lee, J., Hu, Y., Libutti, S. K., Alexander, H. R., and Bartlett, D. L. Complex interactions between the replicating oncolytic effect and the enzyme/prodrug effect of vaccinia-mediated tumor regression. *Gene Ther.*, **7**: 1217–1223, 2000.
41. Hermiston, T. Gene delivery from replication-selective viruses: aiming guided missiles in the war against cancer. *J. Clin. Invest.*, **105**: 1169–1172, 2000.
42. Consalvo, M., Mullen, C. A., Modesti, A., Musiani, P., Allione, A., Cavallo, F., Giovarelli, M., and Forni, G. 5-Fluorocytosine-induced eradication of murine adenocarcinomas engineered to express the cytosine deaminase suicide gene requires host immune competence and leaves an efficient memory. *J. Immunol.*, **154**: 5302–5312, 1995.
43. Ju, D. W., Tao, Q., Cheng, D. S., Zhang, W., Zhang, M., Hamada, H., and Cao, X. Adenovirus-mediated lymphotactin gene transfer improves therapeutic efficacy of cytosine deaminase suicide gene therapy in established murine colon carcinoma. *Gene Ther.*, **7**: 329–338, 2000.
44. Perrelet-Carle, V., Baque, P., Gavelli, A., Mala, M., Chazal, M., Gugenheim, J., Bourgeois, A., Milano, G., Staccini, P., and Rossi, B. Cytosine deaminase/5-fluorocytosine-based vaccination against liver tumors: evidence of distant bystander effect. *J. Natl. Cancer Inst.* (Bethesda), **91**: 2014–2019, 1999.
45. Dilber, M. S., and Smith, C. L. Suicide genes and bystander killing: local and distant effects. *Gene Ther.*, **4**: 273–274, 1997.
46. Ramesh, R., Maraghi, A. J., Munshi, A., Abboud, C. N., and Freeman, S. M. *In vivo* analysis of the "bystander effect": a cytokine cascade. *Exp. Hematol.*, **24**: 829–838, 1996.
47. Huber, B. E., Austen, E. A., Richards, C. A., Davis, S. T., and Good, S. S. Metabolism of 5-fluorocytosine to 5-fluorouracil in human colorectal tumor cells transduced with the cytosine deaminase gene: significant antitumor effects when only a small percentage of tumor cells express cytosine deaminase. *Proc. Natl. Acad. Sci. USA*, **91**: 8302–8306, 1994.
48. Fuchs, E. J., and Matzinger, P. Is cancer dangerous to the immune system? *Semin. Immunol.*, **8**: 271–280, 1996.
49. Matzinger, P. An innate sense of danger. *Semin. Immunol.*, **10**: 399–415, 1998.
50. Kiehl, E., Nyati, M. K., Ng, E., Stegman, L. D., Parsels, J., Ross, B. D., Rehentulla, A., and Lawrence, T. S. Yeast cytosine deaminase improves radiosensitization and bystander effect by 5-fluorocytosine of human colorectal cancer xenografts. *Cancer Res.*, **60**: 6649–6655, 2000.

What are the reasons for negative phase III trials of molecular-target-based drugs?

Nagahiro Saijo

National Cancer Center Hospital East, 6-5-1 Kashiwanoha, Kashiwa-shi, Chiba 277-8577

(Received May 12, 2004/Revised July 1, 2004/Accepted July 23, 2004)

The results of molecular-biological studies of cancer are changing the way we diagnose and treat cancer. Target-based drug discovery selects agents for development based on their mechanisms of action. The interaction between target-based drugs and their targets can be described by classical drug-receptor theory. Clinical trials have demonstrated that some effective target-based drugs induce apoptosis, even though they are considered to be cytostatic. Numerous phase III trials of target-based drugs have been conducted. Although some have yielded strongly positive results, the majority of the results have been negative. This article seeks to clarify the value of molecular-target-based therapy and to discuss the reasons for negative results in phase III trials. The importance of proof-of-principle studies is stressed throughout preclinical and clinical trials of molecular-target-based drugs. (Cancer Sci 2004; 95: 772-776)

In an attempt to modulate specific molecular targets in tumor cells and the tumor environment, the emphasis in anticancer drug discovery has shifted from an empirical approach characterized by random screening of a variety of natural and synthetic compounds by means of cell-based cytotoxicity assays to more rational and mechanistic molecular screening.¹⁻⁹⁾

The concept of molecular-target-based therapy is not new, because modern therapy of breast cancer and prostate cancer is in reality target-based therapy. What is new is that we now recognize that tumor cells contain many targets, and drugs specifically directed at such targets have been introduced clinically.⁶⁻⁹⁾

1. Classification and characteristics of molecular-target-based drugs

The molecular-target-based drugs that are currently available can be classified according to mechanism of action into inhibitors of growth factors/receptors and signal transduction, inhibitors of the cell cycle, and inhibitors of metastasis and angiogenesis, and they can further be classified according to site of action into two groups: tumor-specific and tumor-environment-specific. Based on the formulation of the drug products, they can be classified into small molecules and macromolecules (Table 1).

Compared with the process of discovery of empirical drugs, that of molecular-target-based drugs is target-based, and their mechanism of action is the basis for selection. Many researchers consider the pharmacological effect of target-based drugs to be cytostatic/reversible, as opposed to the cytotoxic/irreversible effect of cytotoxic drugs; however, all recently approved antitumor molecular-target-based drugs cause tumor shrinkage except for avastin. Since the effects of molecular-target-based drugs are more selective, the drugs are expected to be less toxic. The clinical data suggest that molecular-target-based drugs have different spectra of adverse events. They are expected to be more effective when given continuously at tolerable doses.^{5, 8)}

2. End points and strategies of clinical trials

It is generally said that the paradigms for the development of molecular-target-based drugs should be shifted from empirical to more scientific. The concept of drugs has shifted from "seek and destroy" to "target and control," and screening has shifted from "random screening against tumors" to "target-based screening against tumor-specific molecules". It is extremely important to evaluate the effect of drugs on their targets pharmacodynamically, although no proof-of-principle studies are required for cytotoxic drugs. The paradigms for other pharmacodynamic effects, such as tumor shrinkage and toxicities, as well as pharmacokinetics have not changed. The primary end point of clinical trials, "survival," is the same as for evaluation of cytotoxic drugs. The major end points for ordinary approval of oncology drugs are survival and response rate (Table 2).

Only one drug, trastuzumab, has been approved based on data showing an increase in time-to-progression. However, trastuzumab itself causes tumor shrinkage and a survival benefit when combined with other anticancer drugs. The initial clinical study for cytotoxic drugs is a phase I study. Phase II and I/II studies follow, and the final conclusive study is a phase III study. Phase II studies are sometimes skipped for molecular-target-based drugs, and the phase III study sometimes follows the phase I study (Fig. 1).

3. Clinical trials of molecular-target-based drugs

Table 3 shows the results of clinical trials of specific-target-based drugs.

Randomized trials have shown positive results for imatinib, trastuzumab, and rituximab.^{10, 11-13)} Imatinib was used as a single agent, whereas trastuzumab and rituximab were evaluated as combination therapy. Table 4 shows the results of clinical trials of non-specific or tumor-environment-specific target-based drugs, which include antiangiogenic and/or antimetastatic drugs. The results of all of the phase III clinical trials were negative, except for the trial of avastin for colon cancer.¹⁴⁾ Even the results of the phase III trial of avastin for breast cancer were completely negative. Generally speaking, antibodies and some small molecules have shown promise. Signal transduction modulators that act upstream of a growth signal have been found to show a survival benefit. All of the compounds approved for commercial sale caused tumor shrinkage when given alone.

4. Reasons for negative phase III trials

Several hypotheses have been proposed to explain the negative results of phase III trials (Table 5).

One is that the molecular target was not essential for growth, invasion, or metastasis of the tumor, and this hypothesis may

E-mail: nsaijo@east.ncc.go.jp

Table 1. Classification of target-based therapies according to molecular targets

1) Inhibitors of growth factors/receptors & signal transduction
Anti-EGFR Ab, Anti-Her-2/Neu Ab, EGFR-TKI, c-Kit receptor TKI, Bcr-abl-TKI, Farnesyltransferase inhibitors, PKC inhibitors
2) Inhibitors of the cell cycle
Cyclin-dependent kinase (CDK) inhibitor
3) Inhibitors of metastasis & angiogenesis
Anti-VEGF Ab, VEGF inhibitor, MMP inhibitor, Thalidomide, Angiostatin, Endostatin

Table 2. Requirements of paradigm shifts for new anticancer drug development

1) Concept and screening	Paradigm shift
Seek and destroy	→ Target and control
Random screening against tumors	→ Target-based screening against tumor-specific molecules
2) PK and surrogate end point	Paradigm shift ? → Target effect
	No paradigm shift
PK	→ PK
Tumor shrinkage	→ Tumor shrinkage
Toxicity	→ Toxicity
3) Primary end point	No paradigm shift
Survival	→ Survival

Table 3. Results of phase III trials of specific-target-based drugs

Agent	Tumor	Combination	Results
ZD1839	NSCLC	Y	Negative
		N	Too early
OSI774	NSCLC	Y	Negative
		N	Positive
STI571	CML	N	Positive
	GIST	N	Positive
Trastuzumab	Breast	Y	Positive
	NSCLC	Y	Negative
Rituximab	NHL	Y	Positive
Affinitac	NSCLC	Y	Negative

NSCLC, non-small cell lung cancer; CML, chronic myelocytic leukemia; GIST, gastrointestinal stromal tumor; NHL, non-Hodgkin's lymphoma; Y, yes (+); N, no (-).

explain the negative results for matrix metalloprotease inhibitors¹⁵⁾ and small molecules against vascular endothelial growth factor (VEGF) tyrosine kinase.

Another hypothesis is that the target tumor contains no, or only a low level of the molecular target. The results of the randomized trial of trastuzumab for the treatment of non-small cell lung cancer (NSCLC) were completely negative,¹⁶⁾ but expression of Her-2 is very low in NSCLC. This explanation is also valid for the results of the trial of imatinib against small cell lung cancer, which expresses only low levels of c-kit.¹⁷⁾ Enrichment of the target population with the molecular target is essential to obtain 'positive' results. These are likely to be the reasons why the molecular-target-based drugs did not exert adequate antitumor activity.

The majority of molecular-target-based drugs tested in the clinical trials have been evaluated in combination with cytotoxic drugs and other modalities. There are many problems with predictability in preclinical models, especially in regard to

Table 4. Phase II* and phase III trials of non-specific or tumor-environment-specific target-based drugs**

Agent	Tumor	Combination	Results
Marimastat	SCLC	N	Negative**
	Pancreas	N	Negative**
	Stomach	N	Negative**
	NSCLC	Y	Negative**
Prinomastat	NSCLC	Y	Negative**
Tanomastat	SCLC	Y	Negative**
SUS416	Colon	Y	Negative**
	Breast	Y	Negative**
Avastin	Colon	Y	Positive**
	Renal	N	Positive*

SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; Y, yes (+); N, no (-).

Table 5. Why have so many phase III trials been negative?

1. Poor predictability of preclinical models, especially of combined effects
2. Target tumor contains no, or a low level of the molecular target.
Enrichment of the target population is inadequate.
3. The "molecular target" is not essential for the growth, invasion, or metastasis of the tumor.
4. Molecular-target-based drugs have inadequate antitumor activity.
5. Clinical decision-making for moving from phase I/II to phase III studies is inappropriate.
6. Clinical study design is inappropriate.
Small sample size
Comparison of 3 drugs vs. 2 drugs

combined effects.¹⁸⁾ The purpose of combination therapy is to increase the spectrum and intensity of the anticancer effect. The criteria for the selection of drugs for use in combinations are: 1) each drug must be independently active against the target tumor, 2) the toxicities of the drugs must not overlap, 3) each drug can be used at its most appropriate dose and schedule, 4) the mode of action of each drug is different, and 5) no cumulative toxicity is observed and the regimen can be administered repeatedly. If an active drug is combined with another active drug, a synergistic or additive effect is expected. The majority of molecular-target-based drugs are used as sensitizing drugs, and a synergistic effect is expected based on biochemical or molecular biological interaction. The purposes of preclinical evaluation of combinations are prediction of synergism of effect and toxicity, demonstration of biochemical modulation, provision of a rationale for clinical combination to the physician/IRB/patient. The conditions required for preclinical studies of combination chemotherapy are demonstration of a synergistic/additive antitumor effect *in vitro*, no increase in *in vitro* toxicity against normal cells, molecular proof-of-principle study for synergism, demonstration of a synergistic/additive antitumor effect *in vivo*, and no increase in *in vivo* toxicity. There are many problems in preclinical prediction of combined effects of anticancer drugs, and the results of preclinical prediction of combined effects have been very poor. Most preclinical data for combination chemotherapy have been obtained after the clinical evaluation. In other words, the purpose of preclinical evaluation is confirmation of a combined effect observed clinically. The reasons for the poor preclinical predictability of combined effects are shown in Table 6.

Decision-making to proceed from phase I/II to phase III studies is quite difficult. For example, a combination of docetaxel and Ly900003 has been tried in NSCLC patients after

prior chemotherapy, but the results showed the same efficacy as for docetaxel alone. Randomized controlled trials comparing paclitaxel and carboplatin with or without Ly900003 have shown no difference in antitumor effect or median survival, and the frequency of grade (Gr) 3/4 thrombocytopenia was significantly higher in the Ly900003-containing regimen.¹⁹ At least four problems can be pointed out in this example. The molecular target of Ly900003 is not essential for tumor growth, invasion, or metastasis, the antitumor activity of Ly900003 is inadequate, the evaluation of the results of the combination phase I/II trial is incorrect, and the target populations of the phase I/II and III trials were different. A trial of oblimersen sodium combined with standard combination chemotherapy consisting of etoposide+carboplatin was conducted in patients with extensive small-cell lung cancer.²⁰ The appropriate dose of oblimersen was determined to be 7 mg/kg with etoposide 80 mg/m² and carboplatin area under the curve (AUC)-5. The response rate was 86% (12/14), and the median survival time was 12.5 months. No Bcl2 suppression was observed in peripheral blood mononuclear cells. Although the data are not interesting, and the results of proof-of-principle study were negative, Cancer and Leukemia Group B (CALGB) initiated a phase III randomized controlled trial to evaluate the efficacy of oblimersen sodium against small-cell lung cancer.

The clinical study design and sample size are decided based on expected differences in antitumor effect between regimens,

the feasibility of the study, and the baseline clinical treatment effect of the control regimen. Possible reasons for the well-known negative results of the Iressa NSCLC Trial Assessing Combination Treatment (INTACT) trial are low response rate to gefitinib, absence of survival benefit of gefitinib, crossover use of gefitinib in the control group, and small sample size.^{21,22} Table 7 shows the sample sizes required for phase III trials. Postulating 1-year survival prolongation in responders, no overall survival, and 1-year median survival prolongation in non-responders, and no crossover of treatment regimens, it would be necessary to accrue 7095×2 patients if the response rate to the molecular target-based drug were 10%. An astronomical number of patients would be needed to obtain positive data (Table 7).

5. Role of surrogate end points

Surrogate endpoints are measurements or signs that are used as substitutes for clinically meaningful end points that directly measure how a patient survives and functions.^{23,24} Changes in a surrogate end point in response to therapy should reflect changes in clinical endpoint. Fig. 2 shows the ideal process for a surrogate end point. Measurement of surrogate effects has been considered essential in the clinical evaluation of molecular-target-based drugs, because it seems very important to dem-

Table 6. Reasons for poor predclinical predictability of combined effects

- 1) Molecular target of each drug undetermined
- 2) *In vitro* problems
 - A. Concentration, incubation time, timing, protein binding
 - B. Target tumor
 - C. Effect on normal cells
 - D. End point of combined effect (evaluation method)
- 3) *In vivo* problems
 - A. Dose, timing
 - B. Target tumor
 - C. Species specificity (metabolism, protein binding)
 - D. Endpoint of combined effect

Table 7. Sample size for phase III studies

RR (%)	MST (months)	2-Year survival (%)	#pts.
0	12.0	12.2	—
10	12.7	16.0	7095×2
20	13.6	19.8	1670×2
30	14.3	23.5	709×2
50	16.4	31.1	232×2

$\alpha=0.05$, $\beta=0.20$.

- 1-Year survival prolongation in responders.
- No survival prolongation in non-responders.
- MST: 1 year in non-responders.
- No crossover.

Ishizuka, personal communication. RR, response rate; MST, median survival time.

Flow chart of clinical trials of target-based drugs

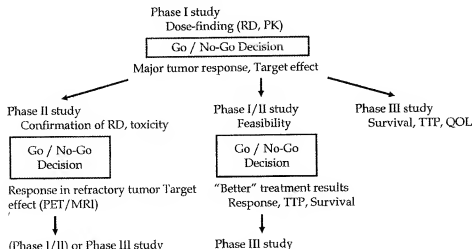


Fig. 1. Flow chart of clinical trials of target-based drugs. Target effects are measured for correlation with effect, dose-finding, and monitoring. RD, recommended dose; PK, pharmacokinetics; TTP, time to progression; QOL, quality of life; PET, positron emission tomography; MRI, magnetic resonance imaging.

Ideal process for a surrogate end point

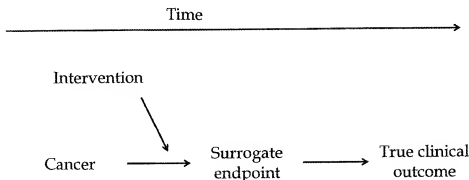


Fig. 2. Ideal process for a surrogate end point. A surrogate end point should be evaluated and obtained before the data of true clinical outcome. It should be a predictor for true clinical outcome.

onstrate that the target effect actually results in a survival benefit. However, many surrogate end points have failed. The reasons can be summarized as follows. The surrogate is not in any of the causal pathways of the disease. There are several causal pathways of the disease, but molecular-target-based drugs affect only the pathway mediated through the surrogate. The surrogate is insensitive to the effect or is not in the pathway of the intervention's effect. The intervention has mechanisms of action that are independent of the disease process. Requirements for target-based therapy are a validated molecular target, reliable assay to measure expression and activity of the target or pathway, difference between target expression in heterogeneous tumor cells, potent and specific inhibitor with good pharmacological properties, and demonstrable target inhibition in human tumors. Valid targets should be expressed, overexpressed, or mutated in tumor tissues. The target should be essential to cell proliferation, cell death, or metastatic ability, and inhibition of the target should result in inhibition of tumor growth and spread. Simple correlates do not make a surrogate. Surrogates can be divided into surrogate-effect end points and surrogate-benefit end points. Surrogate-effect end points involve various problems, such as reliability of the target in tumors, the reliability of the assay method, the tissue specificity of target expression, the extent of heterogeneity, the accessibility of the tissue, and validation against a clinical benchmark. Surrogates for epidermal growth factor receptor (EGFR) inhibition and for anti-angiogenesis are shown in Table 8. Surrogate-benefit end points are objective tumor response, changes in a tumor marker, and changes in tumor metabolism on a positron emission tomography (PET) scan, and they require validation by linkage to a definitive clinical end point in a prospective trial.

6. How can positive data be obtained from phase III trials?

Standard approval of a molecular-target-based drug requires demonstration of a clinical benefit and improvement of the ultimate

Table 8.

Surrogates for EGFR inhibition
1) Rash: skin biopsy
2) EGFR amount: phosphorylation state
3) MAP kinase activation
4) AKT activation
5) Induction of p27 ^{ras}
6) Cell proliferation index
Surrogates for anti-angiogenesis
1) Tumor microvessel density
2) Tumor blood flow (MRI)
3) Tumor metabolism (PET)
4) Tumor apoptosis
5) Circulating endothelial cell apoptosis
6) Circulating VEGF and VEGFR levels

EGFR, epidermal growth factor; MRI, magnetic resonance imaging; PET, positron emission tomography; VEGF, vascular endothelial growth factor.

mate outcome, including improvement of survival, relief of symptoms, or a delay in the onset of symptoms. Appropriate preclinical and early clinical trials are essential to obtain positive results in phase III trials. Scientific decision-making is required. Enrichment of the responsive population based on clinical information and translational research is important. Of course, good and feasible clinical trial designs should be adopted. Urgent requirements include the development of a validated test to define the target population and more effective molecular-target-based drugs to reduce sample size.

Recently a Dana-Farber group reported the identification of EGFR mutations in subset of human lung adenocarcinomas and the association between EGFR mutation and gefitinib sensitivity.

Screening for such mutations in lung cancers may enrich patients who will have a response to gefitinib.^{25,26}

1. Saijo N. The molecular biology and signal transduction of epidermal growth factor receptor. *New Dev Lung Cancer* 2003; 1: 3-6.
2. Sekine I, Saijo N. Growth-stimulating pathways in lung cancer: implications for targets of therapy. *Clin Lung Cancer* 2001; 2: 299-306.
3. Saijo N, Tamura T, Yamamoto N, Nishio K. New strategies for cancer therapy in 21st century. *Cancer Chemother Pharmacol* 2001; 48: S102-6.
4. Schilsky RL. Endpoints in cancer clinical trials and the drug approval process. *Clin Cancer Res* 2002; 8: 935-8.
5. Stadler WM, Ratain MJ. Development of target based antineoplastic agents.

Invest New Drugs 2000; 18: 7-16.

6. Fox E, Curt GA, Balis FM. Clinical trial design for target-based therapy. *Oncologist* 2002; 7: 401-9.
7. Gardner SN, Fernandes M. Cytostatic anticancer drug development. *J Exp Ther Oncol* 2004; 4: 9-18.
8. Saijo N, Tamura T, Nishio K. Problems in the development of target-based drugs. *Cancer Chemother Pharmacol* 2000; 46: S43-5.
9. Saijo N, Tamura T, Nishio K. Strategy for the development of novel anticancer drugs. *Cancer Chemother Pharmacol* 2003; 52: S97-101.

10. Druker BJ, Sawyers CL, Kantarjian H, Resta DJ, Reese SF, Ford JM, Capdeville R, Talpaz M. Activity of a specific inhibitor of the BCR-ABL tyrosine kinase in the blast crisis of chronic myeloid leukemia and acute lymphoblastic leukemia with the Philadelphia chromosome. *N Engl J Med* 2001; 344: 1038-42.
11. Druker BJ, Talpaz M, Resta DJ, Peng B, Buchdunger E, Ford JM, Lydon NB, Kantarjian H, Capdeville R, Ohno-Jones S, Sawyers CL. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N Engl J Med* 2002; 344: 1031-7.
12. Sharon DJ, Leyland-Jones B, Shak S *et al*. Use of chemotherapy plus a monoclonal antibody against Her2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* 2004; 344: 783-92.
13. Coiffier B, Lepage E, Briere J *et al*. CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse B-cell lymphoma. *N Engl J Med* 2002; 346: 235-42.
14. Bevacizumab (anti VEGFAb) prolongs survival in first line CRC. *ASCO* 2003; abstract.
15. Shepherd FA, Giaccone G, Seymour I, Debruyne C, Bezjak A, Hirsh V, Smyth M, Rubin S, Martins H, Lamont A, Krzakowski M, Sadura A, Zee B. Prospective, randomized, double-blind, placebo-controlled trial of mirtazapine after response to first-line chemotherapy in patients with small-cell lung cancer: a trial of the National Cancer Center Institute of Canada-clinical trials group and European Organization for Research and Treatment of Cancer. *J Clin Oncol* 2002; 20: 4434-9.
16. Gatzemeier U, Groth G, Hirsh V *et al*. Gemcitabine/cisplatin alone and with trastuzumab (Herceptin) in patients with non-small cell lung cancer overexpressing HER2: results of a randomized phase II study. *Proc Am Soc Clin Oncol* 2002; 21: 297a (abstr 1185).
17. Johnson BE, Fischer T, Fischer B, Dunlop D, Rischin D, Siderman S, Kowalski MO, Sayles D, Dimitrijevic S, Fletcher C, Hornick J, Salgia R, Le Chevalier T. Phase II study of imatinib in patients with small cell lung cancer. *Clin Cancer Res* 2003; 9: 5880-7.
18. Naruse I, Fukumoto H, Saijo N, Nishio K. Enhanced anti-tumor effect of trastuzumab in combination with cisplatin. *Jpn J Cancer Res* 2002; 93: 574-81.
19. Lynch TJ, Raju R, Lind M, Riviere A, Gatzemeier U, Drorr A, Holmlund J, Yuen A, Sikic B. Randomized phase III trial of chemotherapy and antisense oligonucleotide LY900003 (ISIS 3521) in patients with advanced NSCLC: initial report. *Proc Am Soc Clin Oncol* 2003; 22: 623 (abstr 2504).
20. Rubin C, Kosloff M, Edelman MJ, Hoffman PC, Szeto L, Vokes EE. Phase I study of G3139 (olihomocystine sodium), carboplatin, and etoposide in previously untreated extensive stage small cell lung cancer (SCLC). *Proc Am Soc Clin Oncol* 2003; 22: 631 (abstr 2538).
21. Herbst RS, Giaccone G, Schiller JH, Natale RB, Miller V, Manegold C, Scagliotti G, Rosell R, Offit I, Reeves JA, Wolf MK, Krebs AD, Averbuch SD, Ochs JS, Grous J, Fandi A, Johnson DH. Gefitinib in combination with paclitaxel and carboplatin in advanced non-small-cell lung cancer: a phase III trial—INTACT 2. *J Clin Oncol* 2004; 22: 785-94.
22. Giaccone G, Herbst RS, Manegold C, Scagliotti G, Rosell R, Miller V, Natale RB, Schiller JH, von Pawel J, Pluzanska A, Gatzemeier U, Grous J, Ochs JS, Averbuch SD, Wolf MK, Rennie P, Fandi A, Johnson DH. Gefitinib in combination with gemcitabine and cisplatin in advanced non-small-cell lung cancer: a phase III trial—INTACT 1. *J Clin Oncol* 2004; 22: 777-84.
23. Saijo N. Translational study in cancer research. *Intern Med* 2003; 41: 770-3.
24. Saijo N, Nishio K, Tamura T. Translational and clinical studies of target-based cancer chemotherapy. *Int J Clin Oncol* 2003; 8: 187-92.
25. Paetz JM, Janne PA, Lee JC, Tracy S, Geulich H, Gabriel S, Herman P, Key FI, Lindeman N, Boggan TJ, Naoki K, Sasaki H, Fujii Y, Eck MJ, Sella WR, Johnson BE, Meyerson M. EGFR mutation in lung cancer: correlation with clinical response to Gefitinib therapy. *Science* 1099314, April 30, 2004.
26. Lynch T, Bell DW, Sordella R, Garubhagavata S, Okimoto RA, Brum BS, Brennanigan W, Harris PL, Hascht SM, Sapko JG, Halushka FG, Hazelat SM, Sapko JG, Halushka FG, Louis DN, Christiansi DC, Sattelman J, Haber DA. *N Engl J Med* 2004; 350: 2129-39.

Trials, Tribulations, and Trends in Tumor Modeling in Mice

JOANN C. L. SCHUH

Applied Veterinary Pathobiology, Bainbridge Island, Washington 98110-3663, USA

ABSTRACT

Selection of mouse models of cancer is often based simply on availability of a mouse strain and a known compatible tumor. Frequently this results in use of tumor models long on history but short on homology and quality control. Other factors including genetics, sex, immunological status, method and site of tumor implantation, technical competence, biological activity of the tumor, protocol sequence and timing, and selection of endpoints interact to produce outcomes in tumor models. Common reliance on survival and tumor burden data in a single mouse model often skews expectations towards high remission and cure rates; a finding seldom duplicated in clinical trials. Inherent limitations of tumor models coupled with the advent of new therapeutic targets reinforce need for careful attention to design, conduct, and stringent selection of *in vivo* and *ex vivo* endpoints. Preclinical efficacy testing for anti-tumor therapies should progress through a series of models of increasing sophistication that includes incorporation of genetically engineered animals, and orthotopic and combination therapy models. Pharmacology and safety testing in tumor-bearing animals may also help to improve predictive value of these models for clinical efficacy. Trends in bioinformatics, genetic refinements, and specialized imaging techniques are helping to maintain mice as the most scientifically and economically powerful model of malignant neoplasms.

Keywords. Preclinical efficacy testing; tumor models; study design; xenograft; orthotopic tumors; genetically engineered mice; bioinformatics; bioimaging.

INTRODUCTION

Malignant neoplasms rank second as the leading cause of death in the United States and ranked first in those aged 45-74 (Anderson, 2002). As a result, anti-cancer therapies are a frequent focus for startup companies and represent a major therapeutic class for pharmaceutical, biopharmaceutical, medical device and drug delivery manufacturers. Despite the effort applied to cancer targets, the number of successful new therapies for treating human malignancies is discouragingly low. This is surprising in that many trials are now conducted using novel agents with specificity for molecular pathways and cellular components rather than broad targeting of chemotherapy and radiation to normal and neoplastic cells. Failures in clinical trials are multifactorial with lack of efficacy an important cause. Conversely, modulating and curing experimental cancer in mice is a relatively easy process. Many commonly used mouse models of neoplasia have proven to be biased towards false positive results and preclinical studies have not accurately predicted clinical responses. Therefore, unconditional acceptance of limited data from mouse models has to be avoided to prevent premature movement of development programs into clinical testing. Increasingly, the plethora of novel strategies undergoing testing requires greater attention to proper design and conduct of preclinical efficacy studies. Rationale design of preclinical efficacy studies requires understanding the biology of tumors and implantation techniques, selection of *in vivo* and *ex vivo* endpoints, and a willingness to integrate new and often costly testing strategies that more appropriately mimic the biology of human neoplasms.

BIOMEDICAL MODELS OF NEOPLASIA FOR PRECLINICAL TESTING

Spontaneous and Environmental Carcinogenesis Models

Historically, spontaneous, chemical, ultraviolet (UV), oncogene, and viral infection models helped to define many aspects of carcinogenesis (Harrison, 2002) and therapeutic intervention (Boone et al., 1992) and helped to promote development of inbred strains of mice (Corbett et al., 2002). Despite the significance of spontaneous and environmental models to biomedical research, the long latency of most of these models makes them impractical for most preclinical studies of tumor modulation. Spontaneous, chemical and UV and viral infected or transformed tumors are of greatest importance as the source of many cell lines used for *in vitro* studies and *in vivo* transplantation models.

Transplantation Models

There are many immortalized cell lines of human and murine origin available from commercial sources and privately held by research organizations that have been tested for tumorigenicity in mice (Giard et al., 1973; Gershwin et al., 1977; Fogh et al., 1977; Traimer et al., 1988). In addition to availability, these tumorigenic cell lines are generally easy to maintain, selectable for unique mutations *in vitro* and backed by numerous publications on *in vivo* behavior in immunodeficient (nude, beige, nude/beige, C.B -17 severe combined immunodeficient [SCID], nonobese diabetic [NOD]/SCID), immunosuppressed (thymectomized or corticosteroid treated), humanized (hu)-SCID or hu-NOD/SCID and immunocompetent strains of mice. However, quality control is an issue. Many cell lines have undocumented source and passage histories, poorly characterized receptor and oncogene expression and cellular secretions, and inconsistent designations in publications. Features that make these tumor lines suitable for transplantation may affect experimental design

Address correspondence to: JoAnn C. L. Schuh, Applied Veterinary Pathobiology, 1752 Lewis Place NW, Bainbridge Island, Washington 98110-3663, USA; e-mail: schuhj@bainbridge.net

because of the need for specific mouse strains, sex specificity, and altered host immunity. Furthermore, spontaneous mutations *in vitro* that allow selection of tumor cell line subclones with unique behaviors can also result in point mutations that lead to changes in histomorphology, sensitivity, and behavior of these tumors *in vivo*. Dissociated solid tumors and fragments of tumors (explants) that retain histomorphology relationships of the tumor and associated stroma are also suitable for implantation (Gershwin et al., 1977). Tumor cells from cell lines and solid masses of both human and mouse origin may require handling as biohazardous material due to their histogenesis by viral transformation or inadvertent contamination (Hay, 1991; Nicklas et al., 1993). The literature on tumors that have been used in mice to model important therapeutic targets in humans is vast. Due to the rapidly changing nature of biomedical research, a thorough and current literature search is warranted prior to selecting the most appropriate models for any therapeutic development program. A selection of representative mouse models (transplantation and genomic-based) for malignant neoplasms causing death in humans is provided in Table 1.

Criteria other than historical usage, and availability of the tumor line and a suitable mouse host need to be considered when testing efficacy of tumor modulation with therapeutics (Skipper, 1968; Huseby, 1969; Klausner, 1999). Even with genetically engineered models, multistep progression and clonal derivation of tumors in humans are difficult to model in mice. Therefore, foibles of these models need to be understood to prevent overinterpretation of positive or negative results.

CONSIDERATIONS IN SELECTING TUMOR MODELS IN MICE

It is well recognized that our understanding of modeling of tumor biology and therapeutic intervention is constrained by many factors (Siemann, 1987; Rew, 2000a; Rew, 2000b). However, validation studies of these model systems for their ability to adequately predict therapeutic responses in patients have been rare (Hann and Balmain, 2001). Consequently, design and interpretation of preclinical studies for tumor modeling must be undertaken carefully.

Study Design

Candidate anti-tumor agents can be identified and selected using a broad panel of *in vivo* tumors (Atassi et al., 1988) but false positive and negative results may occur due to incompatible host-tumor-therapeutic interactions or technical incompetence. In efficacy studies, greater depth needs to be achieved through evaluation of at least several subtypes of the tumor representative of the clinical target, and through use of different models systems in mice (transplantation, genetically engineered, and orthotopic models) as well as use of models in other appropriate species. Conduct of animal studies that mimic expected exposure, scheduling, and duration of therapeutic and posttreatment periods of clinical studies are valuable designs prior to initiating clinical trials. Optimization of therapeutic dose and schedule through pharmacokinetic studies are also important early procedures that should be evaluated in one or more model systems. Efficacy studies should also incorporate histopathology endpoints to confirm expected therapeutic target and to help refine dose scheduling relative to growth and cell loss fractions.

Investigators need to be aware that many historical and commonly used model systems in mice were originally established and optimized for use in mechanistic studies (Burger, 2000). Conduct of efficacy testing in such systems has not been optimized and inadvertent selection of systems with excess curability, spontaneous regressions or failure to establish adequate tumor burden (Corbett et al., 2002) can lead to overly optimistic projections of clinical success. Bias in the selection of the model(s) may also arise from experience, knowledge base and objectives of the investigator. Whereas pharmacologists, immunobiologists or cell biologists may consider direct intratumoral injection of therapeutics acceptable, needle tracks and pressure-induced necrosis (compartment syndrome) may interfere with adequate evaluation of such models by a histopathologist. Conversely, intratumoral administration of light activated substances is a common and appropriate route for photodynamic therapy of tumors (Casas et al., 1999). Design of efficacy studies for combination protocols is often difficult. However, such studies can provide important efficacy data for the transition between novel therapies and standard treatment practices that may

TABLE 1.—Rank order of deaths due to malignant neoplasms in the United States in 2001 (Arias and Smith, 2003) and selected published reviews on applicable mouse models of these neoplasms.

Rank	Malignant Neoplasms	Cases	Selected Mouse Models
1	Lung—trachea	156,005	Tuveson and Jacks, 1999; Malkinson, 2001; Liu and Johnston, 2002
2	Colon—rectum	56,799	Heyer et al., 1999; Kobayashi-Larsen et al., 2000; Horig et al., 2001; Boivin et al., 2003
3	Lymphoid—hematopoietic	56,350	Dykes and Waud, 2002; Uckun and Sensel, 2002; Vanderkerken et al., 2003
4	Breast	41,844	Hutchinson and Muller, 2000; Cardiff, 2001; Rosner et al., 2002; Clarke, 2002
5	Prostate	30,714	Royal et al., 1996; Navone et al., 1999; Abate-Shen and Shen, 2002; Nyska et al., 2002
6	Pancreas	29,723	Fu et al., 1992; Hotz et al., 2000; Standop et al., 2001; Bardesy et al., 2001
7	Ovary	14,361	Rahman et al., 1998; Rahman and Hultaniemi, 2001; Orsulic et al., 2002
8	Liver	13,263	Fausto, 1999; Feitelson and Larkin, 2001
9	Brain—meninges	12,567	Holland, 2001; Reilly and Jacks, 2001; Begemann et al., 2002; Gutmann et al., 2003
10	Esophagus	12,509	Opitz et al., 2002
11	Stomach	12,340	Funakawa et al., 1993b
12	Bladder	12,115	Eto et al., 2000; Bonfil et al., 2002
13	Kidney	12,084	Naito et al., 1987b; An et al., 1999; Hillman, 2002
14	Oral	7,638	Waters et al., 1998; Myers et al., 2002
15	Skin	5,543	Alvarez, 2002; Eccles, 2002; Carson III and Walker, 2002
16	Uterus	6,835	Couse et al., 1997; Keshavarzi et al., 2002
17	Cervix	4,064	Herber et al., 1996
18	Larynx	3,826	Chen et al., 2001; Kennel et al., 2002

include a combination of surgery, chemotherapy or radiation, and other supportive therapies (Bogden et al., 1974; Corbett et al., 1979). In designing tumor model systems, investigators must guard against template designs that ignore inherent differences in model systems, and in using insufficient animals per group. This is particularly important in investigational new drug (IND) enabling studies where more animals per group and few groups may be required to adequately control for variation in tumor burden and therapeutic responses. Consultation with a biostatistician to assist in determination of sample size appropriate to each model system is highly recommended.

Context of the Study and Available Resources

Therapeutic intervention conducted in tumor-bearing animals may occur as proof of principal studies for discovery, target validation, and in vivo pharmacology, or as investigations for therapeutic efficacy, safety, and interactions. These types of studies often need to be designed and conducted differently than studies focusing on mechanistic studies. Additionally, an available supply of suitable strains of mice and investigators capable of providing necessary manipulations (injections, surgery, polytherapy, evaluations, and pathology services) may materially affect both quantity and quality of data that can be generated from selected models.

Lack of Quality Control

Investigators using cell lines should develop and maintain in-house databases that allow ready access to known features for tumor cell lines in their collections. A minimum list might include all applicable information that defines origin (human or animal), sex and strain of original host, treatment history (particularly human lines), name and subclone of the tumor line, source (commercial or private), passage history of all aliquots, species, strain and sex and routes susceptible to implanted tumors, in vitro and in vivo growth characteristics, growth rate to maximum humanely accepted and lethal sizes, histomorphological characteristics, metastatic potential and method, immunogenicity, receptor and oncogene expression, cellular products, microbiological screening history, biohazard potential (generally viral) and unique characteristics. This information is invaluable for preventing and investigating problems such as implantation failures, excess curability, microbial contamination, mislabeled or cross-contaminated stocks, and alterations in histomorphology and behavior that may arise from haphazard use of cell lines (Corbett et al., 2002). With rare exception (Rosner et al., 2002), thorough characterization of comparative histology of tumor models using authenticated stocks has not been adequate. Additionally, microbial and cross-species contamination of cell lines has led to erroneous conclusions in tumor biology (Moseley et al., 2003; Drexler et al., 2003). These problems are compounded by lack of source and passage history information reported by most laboratories conducting either mechanistic or efficacy studies. This is not a trivial problem, as the histomorphology and behavior of tumors grown from stocks in different laboratories may eventually differ from historical photomicrographs and behavior descriptions of the original tumor and its cell line (Schuh, unpublished data). Although the original passage history may not be known, investigators should strive to use contaminant free tumor lines of a con-

sistent number of passages from an authenticated stock for preclinical testing. In addition to providing a known in-house passage history, testing from a stock provides a baseline and helps to reduce the potential for point mutations that may cause inter-study variation or erroneous test results.

Paraneoplastic Syndromes

Paraneoplastic syndromes typically result in clinical manifestations of altered physiological responses to autotrophic (spontaneous) neoplasms. These syndromes in mouse models of neoplasia have been seldom described (Liebelt et al., 1974) even though they exist. This oversight is brought on by the focused and short-term nature of most studies. Unlike safety studies where a complete set of tissues are collected and a clinical pathology examination conducted, efficacy studies are often completed without benefit of histopathology and clinical pathology examinations. This type of study design neglects important interactions of the host with tumor receptor expression and secretions produced by tumor cells or by stimulation of host cells by the tumor. Paraneoplastic syndromes include extramedullary hematopoiesis, bone marrow hyperplasia, peripheral granulocytosis and leukocytosis (leukemoid reactions), thrombocytosis, anemia, altered lipid metabolism, hypercalcemia of malignancy, hypoglycemia, cachexia and organomegaly in nontumor-bearing tissues (Liebelt et al., 1974; Castillo et al., 1982; Yoneda et al., 1991; Tanaka et al., 1996; Diamant et al., 1998; Schuh, unpublished data). Paraneoplastic syndromes represent potential models for similar syndromes in humans but causation are poorly characterized and effects of these syndromes on pharmacology, safety, and efficacy studies using experimental tumors are unknown.

Transplantation Protocols: Sites of Implantation Can Affect Study Outcome

Autogenic or autochthonous tumors are seldom practical for tumor modeling for therapeutic intervention. Most transplantable tumors are placed heterotopically (ectopically) in syngeneic (same species, genetically identical), allogeneic (same species, genetically different) or xenogeneic (different species and genetics) host systems. Tumor lines in use have been specifically selected for mutations that allow heterotopic growth in mice. Although these tumors will grow and respond to therapeutics, heterotopic sites are not ideal and selection of the transplantation site may modulate tumor growth (Naito et al., 1987a; Corbett et al., 2002) and success of therapeutic intervention (Averbook et al., 2002).

Subcutaneous (SQ) and less frequently intradermal areas are used for primary tumors for reasons of accessibility, lack of distress and interference with mobility in mice, and visibility for monitoring. Generally, SQ refers to placement by injection or surgical implantation in the flank, a region referring to the posterior lateral abdominal quadrant. Some investigators erroneously include the hindlimb, back and axillary region in their description of the flank. Placement is generally done in fat and mammary gland tissues near popliteal, inguinal or accessory axillary lymph nodes. Despite the common use of SQ sites, it is important to note that even large tumors rarely, if ever, metastasize after implantation in this site (Gershwin et al., 1977; Eccles, 2002). Implantation in the hindlimb (including popliteal lymph node

fat) is sometimes considered superior to placement near body cavities for improved visualization and avoidance of accidental intra-peritoneal or -thoracic implantation. Rarely, tumors may not be viable after SQ implantation and other sites may be required. Implantation of tumors into footpads is generally not acceptable for humane considerations (UKCCCR, 1998; Wallace, 2000). Intramuscular implantation is not common as cell lines and tumor explants may not grow well in muscle compared to SQ, leg mobility is restricted by large masses, expansion space is limited, measurements with calipers are more difficult and this site appears to be more prone to self-mutilation and a target for cage mate aggression. Fat pads other than classic SQ sites, including retroperitoneal, epididymal, intrascapular and mediastinal/thymic sites are also valuable locations for injection and surgical implantation to provide a highly vascular milieu that appears to assist in establishment of xenogeneic tumors. A comparison of tumor viability in brown fat in interscapular areas, compared to the predominate white fat in other fat pads does not appear to have been made.

Primary and sometimes metastatic models are also established by direct surgical or percutaneous intra-organ injections into spleen, liver, lymph nodes, mammary gland, prostate, base of the tongue (head and neck carcinoma), intraluminal (bronchial, urinary bladder, thorax), cecal wall implantation (colorectal metastases), and kidney capsule. Injection into brain and eye are often not considered acceptable by institutional animal care and use committees (IACUC) (UKCCCR, 1998; Wallace, 2000).

Metastatic and sometimes primary tumor models are created by intravenous, intracardiac (ventricle), intraosseous injections or intravascular injections proximal to organs (e.g., portal vein for liver neoplasms). Despite careful preparation to reduce cell clumps and to inject slowly, such tumors are embolic and may grow in unintended sites. Most tumors show selective tissue tropisms, but tumors with broad tropism (e.g., lymphoma/leukemia) may grow within all tissues and result in large unmonitorable tumor burdens (Schuh, unpublished data). Investigators rarely evaluate tissues for metastases outside of their specific area of interest (most often lung and bone) so that characterization of additional tumor burdens and thromboembolism of tumor cells on validity of these tumor models is lacking. Intraperitoneal injections of tumors, once a common route for leukemias and metastatic models, have humane considerations that need to be considered (UKCCCR, 1998; Wallace, 2000).

Implantation techniques must be practiced, as accidental injection into muscle masses or visceral cavities, and post-surgical inflammation can cause intra-study variability and lack of interstudy reproducibility. Migration through SQ tissues or leakage of cell suspensions is generally not an issue with proper technique and minimal volumes injected with a small gauge needle. Interstudy reproducibility of implanted tumor burdens may be affected by extent of cell separation, particularly for disruption of *in vitro* cultures or solid tumors (An et al., 1999). In some efficacy studies, surgical implantation of hollow fibers (Sadar et al., 2002), matrigel and discs (Eccles, 2002), polymers (Righi et al., 2003), liposomes (Kunstfeld et al., 2003), and transparent windows (Dellian et al., 1996; Li et al., 2000; Jain et al., 2002) can provide a tightly contained tumor environment to optimize

reproducibility for certain endpoints such as angiogenesis. Inflammation and interference with tumor biology must be considered when utilizing surgically implanted devices for containment of tumor masses.

Other Host-Tumor-Therapeutic Interactions

Differences in tumor burden potential, angiogenic and therapeutic response have been shown to be due to differences in strains of mice tested (Naito et al., 1987b), primary implantation site and interval between tumor implantation and therapeutic manipulations (Wilmanns et al., 1992; Chakrabarty et al., 1994; Averbook et al., 2002; Monsky et al., 2002), and metastatic microenvironments (Averbook et al., 2002; Seki et al., 2003). For xenogeneic and syngeneic tumors that require immunodeficient mice, differences in immunological defects between these strains should be understood. Similarly, immunogenicity of tumors in immunocompetent hosts may be an important modifying factor. Other factors that can affect tumor growth *in vivo* include concurrent manipulations such as surgery, radiation or concurrent treatments that produce inflammation and inhibit establishment of tumor cells. Conversely, stress and seasonal effects may increase tumor burden and distribution (Gibaldi et al., 2000). Finally, for long standing tumors, tumor-induced cachexia may suppress tumor growth and enhance efficacy (Laster et al., 1961; Chakrabarty et al., 1994; Mukherjee et al., 2002), similar to delays in tumor development found with intentional caloric restriction in rodents (Sutcliffe et al., 2003). Therapeutic efficacy is also modulated through tissue specific and immunologic modifiers, and differences in drug metabolism and disposition (Gershwin et al., 1977; Naito et al., 1987a; Wilmanns et al., 1992; Averbook et al., 2002; Seki et al., 2003).

Specific strains of mice used for tumor models are usually matched to origin of the transplanted tumor. Where multiple tumor cell lines are available to model certain tumors, unique characteristics of these tumors such as host origin, oncogenes, receptors and secretions, and tumor stage may be an important consideration. However, these parameters are often difficult to match to comparable stages in humans (Harrison, 2002). With these limitations in available models, selection of or reporting only on tumors that are dramatically and positively affected by test therapeutics should be avoided. Testing therapeutic interventions on similar tumor types derived from multiple cell lines provides a test situation that partially addresses heterogeneity of tumors in humans. That some of these tumors may not be modulated by the test therapeutic should be expected, and these nonresponders should not be treated as preclinical failures or dismissed. Rather, both negative and positive tumor responses can provide investigators with a more realistic expectation of therapeutic potential in humans, and the totality of the response across several models provides superior insight into activity of therapeutics.

ENDPOINTS AND EVALUATION CRITERIA FOR TUMOR MODELS *IN VIVO*

Critical host-tumor-therapeutic interactions, dose-response, treatment protocol design, and selection of endpoints interact to produce outcomes in tumor modeling (Skipper, 1990; Kerbel, 1999). The goal in clinical oncology is regulation to improve survival and quality of life, and

to prevent recurrent disease rather than to cure/kill cancer (Schipper et al., 1995). More stringent criteria than curability of experimental tumors in mice need to be assessed to determine therapeutic efficacy. Counts of tumor-bearing animals and measurement of tumor burden are the easiest and most frequently used outcomes of efficacy in preclinical studies. In reality, endpoints need to be matched to type of tumor (solid, leukemia or metastatic), context of the study, accessibility of the implantation site, type of implantation, and therapeutic class. Simplistic criteria used in mouse models do not match criteria of partial and complete responses used in clinical oncology, and they contribute to the conflicting opinions about the relevance and predictability of mouse models. Therefore, use of other metrics, and evaluations of angiogenesis, immunomodulation, metastases, and detailed histopathology need to be incorporated into most study designs for preclinical testing. Despite the technical difficulty, labor-intensive nature, and expense commonly cited as limitations for detailed examinations, the utility of mouse models is improved by multiple and appropriate endpoints (Table 2).

In Vivo Endpoints

Tumor growth inhibition studies where treatment is physiologically administered before or on the day of tumor induction are not realistic for preclinical evaluation of clinical responses. Typically, preclinical efficacy studies utilize tumor growth delay in which tumors are induced by injection or surgical implantation and allowed to establish for a number of days prior to initiation of treatment. Solid tu-

mors in accessible sites are amenable to a variety of metrics that are not applicable to primary and metastatic tumors of internal organs and hematologic neoplasms. A count of tumor-bearing animals assumes that nontumor-bearing animals represent tumor regression or cures. Spontaneous regressions, failure of tumors to become established or displaced tumor mass into adjacent body cavities may account for a small percentage of false cures. For this reason, tumor onset and progression should be monitored daily to ensure adequate and similar tumor masses prior to treatment and monitor onset of regression in each group. In models with log-phase tumor growth, animals can be randomized into treatment groups after a predictable period of development, usually 3–14 days. Conversely, less well-developed tumor models may require enrollment of individual animals into the study when the tumor burden reaches a minimum size. Such enrollment studies are difficult to evaluate. Allowing extra days for tumor growth may be as misleading as starting treatment on small tumor masses that have not established and show enhanced regression after onset of treatment. In vivo progression of tumor burden should be evaluated on a daily basis, excluding tumor-free animals as they appear. Burden is commonly measured with calipers and volume estimated from measurement of two (length and width) dimensions (Corbett et al., 2002; Teicher, 2002). Estimates of tumor weight (length × width²/2) using the typically inaccurate measurements derived from tumors of varying shapes and boundaries is not recommended. Tumor growth delay measures the difference in days for the mean or median tumors in test and control animals to reach a specific volume, usually

TABLE 2.—Endpoints to evaluate preclinical efficacy of tumor models in mice.

Endpoint	Comment	Formula ^a
In vivo		
Tumor onset	Day of palpable tumor mass of preselected size	
Tumor progression	Daily plot of tumor burden	
Number of tumor-bearing animals	Tumor-free assumes cure	
Tumor burden—in vivo	Measured (mm ³) Volume estimated (mm ³)—2-dimensional measurement	Length × width Length × (width × 2) ^b Length × (width ²) × 0.5 T – C in days 1) (T – C in days)/3.32 × T _d 2) (T – C) (duration of treatment in days)/3.32 × T _d T/C (%)
Tumor growth delay	Delay to reach specific volume	
Tumor cell kill	1) Log ₁₀ total tumor cell kill 2) Net Log ₁₀ tumor cell kill	
Survival—life span or long-term	Increase in mean or median lifespan or long-term survivors	
Ex Vivo		
Survival—number alive	1) Treatment termination 2) Posttreatment	
Tumor burden—gross pathology	1) Volume estimated (mm ³)—2- or 3-dimensional measurements 2) Absolute weight (mass in mg as water displacement or wet weight) 3) Tumor weight relative to body weight 4) Ulceration 5) Invasion or tissue distribution and gross lesions (e.g., infarction)	Length × width × depth Tumor weight/body weight
Hematology	1) Complete blood count 2) Differential blood count 3) Bone marrow differential	
Histopathology	1) Hematoxylin and eosin a. Confirm histogenesis and differentiation b. Identify invasion and metastases c. Confirm expected therapeutic activity d. Necrosis—characteristics and estimate percentage e. Evaluate angiogenesis, hemorrhage, edema, and immune and stromal responses 2) Morphometrics 3) Immunohistochemistry and in situ hybridization 4) Molecular pathology	

^aT = test; C = control; T_d = doubling time; 3.32 = doublings to increase 1 log₁₀ unit.

^bFormulas used for volume estimates vary.

0.5–1.0 cm³. This value has been suggested to mimic clinical endpoints and disease progression, but this measure is often incorrectly applied. A 50% reduction in tumor mass as a measure of cytoreduction used to gauge clinical responsiveness is not equivalent to 50% inhibition of tumor growth commonly used as a measure of preclinical efficacy (Corbett et al., 2002; Teicher, 2002). Tumor cell kill (net and total) for leukemic or solid tumors requires tumor titration using 10-fold dilutions to determine doubling time, and comparison to tumor growth delay and duration of treatment. Despite the value and more exact nature of these determinations, extra time and additional test animals required generally result in infrequent use of these endpoints (Corbett et al., 1999; Harrison, 2002; Teicher, 2002). Survival data should not be mistaken for mortality, an unacceptable endpoint (UKCCCR, 1998; Wallace, 2000). An increase in percentage mean or median survival may be measured for the lifespan or in long-term survivors. Clinically, posttreatment survival is a valuable endpoint that evaluates treatment efficacy during and after treatment. Survival at study termination is inadequate due to the short duration of most assays and lack of posttreatment data. For many tumors, a log-linear growth cannot be assumed, and more importantly, regrowth of tumors post-treatment may escalate due to increased doubling times (Gunduz et al., 1979; Teicher, 2002). Retention of a subset of animals for several weeks after treatment ends may demonstrate a rapid regrowth of tumor at the efficacious dose that quickly parallels morbidity of controls and lower dose groups. In spite of a delay in tumor growth over the treatment period, a rebound effect post-treatment would be an indication of lower overall efficacy.

Ex Vivo Endpoints

At study termination, volume measurements similar to those performed *in vivo* can be made at gross pathology, but mass (water displacement) or absolute wet weight *ex vivo* is more exacting. Volume measurements at termination may show significant disparity from final *in vivo* measurements. Irregular shapes, small or multilobular masses and necrosis, edema, and hemorrhage all contribute to variation in estimates of tumor burden measurements. Relative tumor to body weight ratios are often useful to gauge therapeutic efficacy in slow growing tumors and when cachexia is present. The relative ratio can reduce or expand differences between controls and treated animals and is a valuable and easily obtained supplement to absolute tumor weight. Visualization and quantification of metastases is particularly difficult to monitor as multifocal and occult tumor burdens are frequent. Flooding of airways with India ink to highlight lung masses for counting, "bread loafing" of the target organ for metastatic counts and organ weight differences compared to controls are inexact measurements prone to intra- and inter-study variability. Such models are better served by tagged tumors and advanced imaging technologies. Gross pathology examination should evaluate presence or absence of ulceration, extent of intentional or inadvertent invasion, tissue distribution beyond the primary implant site, and identify other gross lesions. Accidental implantation of tumors into body cavities can skew results by partial growth of tumor at the intended site that responds to treatment, along with a large internal tumor burden that is unresponsive to treatment. Hematology

(complete blood count and differential) and bone marrow differentials (smears or fluorescent activated cell sorting analysis) can provide data about paraneoplastic syndromes and toxicity. However, hematology and histopathology endpoints are often considered elective. Histologic screening of tumors is valuable to confirm histogenesis and state of differentiation of tumors for quality control and to identify and separate local invasion from metastasis. Characteristics of the tumor such as necrosis, angiogenesis, hemorrhage and immune cell and stromal responses should be monitored. More importantly, histology should confirm the expected activity and target of the therapeutic class. Novel therapeutics currently under development are driving the need for additional *ex vivo* studies such as morphometry to evaluate angiogenesis and apoptosis, immunohistochemistry, *in situ* hybridization and molecular pathology to evaluate changes in macromolecules, immunophenotypes and nucleic acids.

Pharmacology and Safety Endpoints in Tumor-Bearing Animals

Toxicokinetic (TK), and absorption, distribution, metabolism, and excretion (ADME) studies are generally performed early in drug discovery (Lin et al., 2003) and are often incorporated into anti-tumor protocols to determine dose and scheduling. Conversely, safety testing is almost exclusively performed in nontumor-bearing animals. Considering the altered homeostasis of most tumor-bearing animals and humans, safety in a tumor-bearing host should be considered to fully evaluate the therapeutic response and potential. Adverse events can be monitored using a standard safety protocol in tumor and a non-tumor-bearing mouse model, but tolerability of treatment is more difficult to monitor. Limited resources and economic restraints would not support conduct of a full safety program in tumor-bearing animals, but examination of a full panel of tissues from at least one model may be useful to identify adverse effects, modulation of paraneoplastic syndromes, and tolerability issues. Although safety information is generally collected in immunocompetent animals, use of immunodeficient strains may be a better predictor of TK/ADME and safety as a mimic of immune dysfunction after chemotherapy, radiation therapy, surgery (Wichmann et al., 2003), infections and paraneoplastic syndromes (Tanaka et al., 1996). A modified safety study can be incorporated into an efficacy study by collecting a complete or selected list of tissues from tumor-bearing animals and by including treated but non-tumor-bearing animals as additional controls. Although safety information in tumor-bearing animals may be incomplete, this data supplements efficacy, TK and ADME data and can provide needed redirection in dosage and scheduling for subsequent studies.

Humane Considerations

Therapeutic intervention trials may use both short-term and long-term tumor growth models depending on growth rate and aggressiveness of the tumor. Mortality should not be used as an endpoint. Commonly used endpoints such as tumor burden should be limited according to absolute values and relative to body weight. Therefore, careful monitoring and humane endpoints need to be developed in conjunction with the IACUC. Ulceration, tissue and body cavity distension,

inanon, cachexia, anemia, increased intracranial pressure, self-mutilation, cannibalism, and metastases also contribute to premature death of study animals and may require a limited protocol or modified study design (UKCCCR, 1998; Wallace, 2000).

TRENDS IN TUMOR MODELING

Bioinformatics and Mouse Models of Cancer

The evolution of new mouse models of cancer is tightly bound to knowledge gained from sequencing of human and mouse genomes, and compilation and dissemination of this knowledge on the World Wide Web. Important databases for tumor modeling include the Mouse Tumor Biology Database at (<http://tumor.informatics.jax.org>) (Naf et al., 2002), databases accessible through the National Center of Biotechnology (<http://www.ncbi.nlm.nih.gov>) including human and mouse genomes (Wheeler et al., 2003) and The Whole Mouse Catalog (<http://www.rodentia.com/wmc/>).

Technical Trends in Tumor Modeling

Technical advances in tumor modeling have included use of skin fold and transparent windows (Dellian et al., 1996; Li et al., 2000; Jain et al., 2002), matrigel (Eccles, 2002), polymers (Righi et al., 2003), liposomes (Kunstfeld et al., 2003), chambers (Dvorak et al., 1987) and hollow fibers (Sadar et al., 2002) to contain tumor growth and monitor angiogenesis. Ex vivo perfusion (Kristjansen, 2002) and intravital microscopy are providing insights into drug metabolism, angiogenesis and in situ tumor responses (Jain et al., 2002).

Improved imaging of tumors has revolutionized noninvasive monitoring of distribution, growth, metastasis and morphometrics of tumor models in mice. Available systems include micro positron electromagnetic imaging (Ray et al., 2003; Yang et al., 2003), magnetic resonance imaging (Berr et al., 2003; Nelson et al., 2003), in situ visualization of primary and metastatic tumors and occult metastases (Menon and Teicher, 2002), using improved fluorochromes (Rosenberg et al., 2003) and quantum dots (Watson et al., 2003), green fluorescent protein (GFP) (Hoffman, 2002), and luciferase (Edinger et al., 1999; Burgos et al., 2003). Improved fluorochromes, GFP, and LacZ (Kruger et al., 1999; Culp et al., 2001) are also retained within tissues and allow microscopic evaluation by fluorescent microscopy and histochemical or immunohistochemical staining of tumor tissues. Laser capture microdissection is a proven technology for identification of genetic heterogeneity of tumors that continues to expand our knowledge of tumor biology (Culp et al., 2001; Hoon et al., 2002). Specialized microscopy using confocal (Paddock, 1999) and deconvolution (Maierhofer et al., 2003) microscopes are also positioned to provide optical sectioning for identification of 3-dimensional distribution of tumor elements and drug distribution within tumors (Manivasager et al., 2002). While many of these improvements are expensive and impractical for incorporation into preclinical studies, selective use of advanced technologies in drug discovery, pharmacology and preclinical studies can provide minimally invasive and detailed information about host-tumor-therapeutic interactions in vivo at multiple time-points.

Biological Trends: Genetically Engineered Mice

Neoplastic transformation and progression requires a series of genetic alterations that disrupt the balance of cellular mechanisms involving cellular growth and deletion. Mice have played an important role in defining the genetic mechanisms of carcinogenesis. Thus, it is no surprise that genetically engineered mice (GEM) are beginning to take their rightful place as models that show accelerated tumor development and recapitulate the genetics and behavior of human cancer states and cancer resistance (Mickisch et al., 1991; Pitot, 2001; Klatt and Serrano, 2003). Selected target genetic events allow creation of gene-driven gain of function transgenic, loss of function deletion (Reilly and Jacks, 2001; Meuwissen et al., 2001; Resor et al., 2001; Balmain, 2002; Jackson-Grusby, 2002; Herzog and Christofori, 2002; Tuveson and Jacks, 2002; van Dyke and Jacks, 2002), conditional function (Jonkers and Berns, 2002), clones (Rideout III et al., 2000) and phenotypically-driven *N*-ethyl-*N*-nitrosourea mutants (Justice et al., 1999; Balmain, 2002). Although genetically engineered mice have been advocated for preclinical testing (Feitelson and Larkin, 2001; Horig et al., 2001), problems associated with these models include discordance in etiology and histogenesis between human and mouse tumors, multifocal tumors due to multi-tissue deletions or promotor promiscuity, failure to metastasize, variable penetrance of transgenes, long latency (Rosenberg and Bortner, 1999; Moore and Nagle, 2000), limited availability, lack of extensive historical pathology databases for founder strains (FVB, 129 strains, BALB/c), and costs and effort in creating and maintaining specialized GEM animal colonies. Regardless, the ability to use GEM to genetically, anatomically, pathophysiologically and histologically mimic tumors found in humans is becoming a reality. As more logistical problems are overcome, these genomic-derived models will likely become more consistently incorporated into preclinical testing. Compared to transplantation models, GEM will be particularly useful to create tumor states that were previously difficult to model including nervous system (Gutmann et al., 2003), pancreas (Hotz et al., 2000; Bardeesy et al., 2001), lung (Tuveson and Jacks, 1999; Liu and Johnston, 2002), breast (Hutchinson and Muller, 2000), ovarian (Rahman et al., 1998; Rahman and Huhtaniemi, 2001; Orsulic et al., 2002), prostate (Sharma and Schreiber-Agus, 1999; Navone et al., 1999), oral (Opitz et al., 2002), liver (Fausto, 1999; Feitelson and Larkin, 2001; Koike, 2002), hematologic malignancies (Bernardi et al., 2002; Herzog and Christofori, 2002), pediatric tumors (Beltlinger and Debatin, 2001; Houghton et al., 2002), gene-environment interactions (Hursting, 1997), and metastases (McClatchey, 1999; Herzog and Christofori, 2002), and to test interventional feasibility for novel therapeutics against bone metastasis (Fausto, 1999), cell adhesion dysfunction (Herzog and Christofori, 2002), telomere dysfunction (Goytisolo and Blasco, 2002; Artandi, 2002; Granger et al., 2002), ribosomal RNA modification (Ruggiero et al., 2003), and DNA hypomethylation (Goodman and Watson, 2002; Gaudet et al., 2003). Despite these ongoing advances in model systems, disease states associated with tumors such as minimal residual disease (Teicher, 1997; Wetterwald et al., 2002), concurrent opportunistic infections, and treatments such as surgical debulking and combination

therapy will continue to resist modeling in mice for practical and humane concerns.

Biological Trends: Modified Immunodeficient Mice

Nude (athymic) and C.B-17 SCID (T and B cell deficient mice) fail to reject engraftment with a variety of human and mouse tumor cells (Pettaway et al., 1996; Lapidot et al., 1997; Bankert et al., 2002; Giovannella, 2002; Uckun and Senses, 2002) by virtue of their T and B cell defects. The presence of innate immunity, particularly natural killer (NK) cell activity is probably an important factor in limiting tumorigenesis and metastases in these models. The nude mutation results in a mouse that is T cell deficient and has B cell maturational defects, but with intact innate immunity including tumoricidal macrophages and an increase in NK cells. This mutation has been introduced into many mouse strains. Introduction of the beige mutation blocks NK and myeloid-derived cell activity and additional B cell defects result by crossing with the X-linked immunodeficiency mouse. The Chédiak-Higashi syndrome (hypopigmentation, bleeding diathesis and recurrent bacterial infections) in mice or hybrids with the beige anomaly limits their usefulness in surgical, including orthotopic models (Clarke, 2002). The T and B cell deficient SCID mouse has been particularly amenable to further manipulation and modifications involving expanded immunodeficiencies and "humanization." These mice are useful for mechanistic studies and for evaluating anti-tumor therapies on human xenografts (Bankert et al., 2001). However, NK cell activity and the tendency of some SCID mice to become "leaky" and develop active T and B cells, and early onset lymphoproliferative diseases can be problematic. Introduction of the nonobese diabetic (NOD) mutation and recombination activating gene (Rag) deficiency are useful modifiers for the SCID mutation (Clarke, 2002; Eccles, 2002). The NOD mouse is a complex model of immune defects that includes autoimmune mediated type-1 diabetes and sialitis, and Rag-1 and Rag-2 null state produces a severe combined immunodeficiency. Combination of these mutations with SCID defects results in mice that are no longer leaky and NK cell activity is reduced while retaining the ability to support xenografts (Bankert et al., 2001).

SCID or NOD-SCID mice made chimeric with implanted human lymphoid tissues, peripheral blood cells or bone marrow cells are the most commonly used "humanized" mouse models. These models have been generated to provide surrogate human microenvironments and to test immunotherapies (Bankert et al., 2001). Anti-tumor responses in humanized mouse models have not been extensively validated and their use supplements rather than supplants other mouse models for antitumor testing.

Biological Trends: Orthotopic Models

Less than ideal growth and behavior of heterotopic implants has lead to development and characterization of surgical and cellular orthotopic implants (Manzotti et al., 1993). Both normal and neoplastic cells (individual or clusters) and histologically intact tissues, of fetal and adult origin can be used in syngeneic and xenogeneic orthotopic mouse systems. Normal tissues can be implanted and mice injected with tumor cells to produce an improved metastatic model, and using tumor cells with a reporter gene allows enhanced imaging

capabilities. Implantation of tumor tissue into anatomically correct tissue for the histogenesis of the tumor (i.e., kidney tumor in kidney) rather than heterotopic implantation promotes improved tumor growth and metastases (Naito et al., 1987a, 1987b; Menon and Teicher, 2002). Orthotopic models also allow correlation of experimental responses with the original tumor in the host (Steel, 1987).

Although orthotopic models have been utilized for many years, the need for surgical manipulation in many of the models and need for a fresh source of human tissues (normal or neoplastic) adds to the difficulty and expense of this type of implantation. Regardless, orthotopic models provide a useful model for establishing tumors previously difficult to model (Fu et al., 1992; Furukawa et al., 1993a, 1993b; An et al., 1999; Myers et al., 2002) and can be used alone or in combination with humanized mice (Klausner, 1999; Kunstfeld et al., 2003). Orthotopic models allow efficacy testing for tumor inhibition and metastases (Menon and Teicher, 2002; Zhang et al., 2002; Boyd et al., 2003) and appear to be better predictors of clinical success than heterotopic models (Kuo et al., 1993; Manzotti et al., 1993; Killian et al., 1999; Hoffman, 1999; Bloomston et al., 2002).

Other Animal Models for Preclinical Efficacy Studies

Their size, fecundity, ease of handling, relatively economical production and care (excluding genetically altered mice), strain selection, and short gestation backed by massive databases of susceptibilities, genetics, immunology, physiology, pathology, and microbiology make mice an ideal candidate for tumor biology and preclinical efficacy studies. Rats and hamsters are also useful for biological and therapeutic modulation because of their propensity to develop a variety of spontaneous tumors, their amenability to tumor implantation, availability of cell lines (Schwartz and Gu, 2002; Thompson and Sporn, 2002), and recent development of genetically altered rats lacking suppressor genes linked to breast and ovarian cancer (Zan et al., 2003). Beyond rodents, a limited number of spontaneous neoplasms in companion and domestic animals (Vail and MacEwen, 1997; Knapp and Waters, 1997; Dewhirst et al., 2002) and other species including genetically altered fish (Vanchieri, 2001; Spitsbergen and Kent, 2003) also have some potential value in preclinical efficacy testing. Dogs, particularly older males provide a very useful model of prostatic disease (Waters and Bostwick, 1997; Waters et al., 1998; Strandberg, 2000) and dogs larger than purpose-bred Beagles are often the only relevant model to test medical devices or device/drug combinations for anti-tumor applications.

TOWARDS AN IDEAL PRECLINICAL EFFICACY TESTING PROGRAM

In order to transition into clinical trials, regulatory agencies must be provided with evidence that the new therapeutic has an improved safety and/or efficacy profile compared to current therapies. Therefore, selection of tumor models in mice is best approached by using specific criteria that match biologic (multistage, clonal, progression, histomorphology, and metastasis), genetic (multiple mutations, altered chromosomes and cell signaling, genetic expression profile and susceptibility), inductive etiology (chemicals, UV light, diet, hormones and viral), immunogenicity and therapeutic

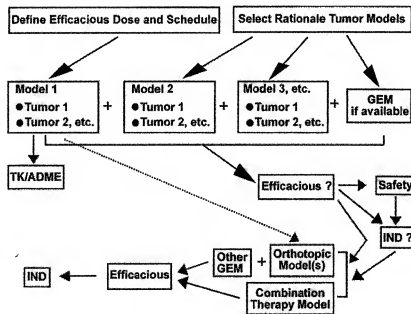


FIGURE 1.—A guide for preclinical efficacy testing of anti-tumor therapeutics. In addition to toxicokinetics (TK) and absorption, distribution, metabolism and excretion (ADME) studies, development programs should progress through an increasingly complex series of tumors in one or more mouse models, genetically engineered mice (GEM) and suitable models in other species. Once a probable level of efficacy is established by use of stringent endpoints in multiple models, an investigational new drug (IND) application may be feasible. Safety studies in tumor-bearing mice should be considered to evaluate toxicity during altered host homeostasis. If insufficient efficacy is established, additional studies utilizing orthotopic models, GEM and combination therapy models may be necessary to provide sufficient evidence of probable clinical efficacy. Well-characterized orthotopic and GEM models may be biologically superior to testing in other models and may provide a shortcut (dotted line) to demonstrating efficacy.

potential (target and predictivity for clinical success) between mice and humans (Siemann, 1987; Hann and Balmain, 2001; Balmain, 2002). Although a generic program is not advocated, *in vivo* preclinical efficacy programs for anti-tumor therapeutics should follow a guide for testing similar to that outlined in Figure 1. After selecting the appropriate dose and scheduling, an efficacy program most economically progresses from simple to more complex tumor model systems. Use of one or more simple models alone to determine possible clinical efficacy is contrary to the complexity and issues associated with tumor models in mice and the variation in accepted therapeutic protocols in clinical oncology. Utilizing multiple surrogate models (cells and explants, different strains of mice and/or additional species, different tumor stages), transplants using variants of the target tumor, and tumor testing in genetically altered animals begins to provide sufficient efficacy data that can support an IND application. This efficacy data should be accompanied by results from PK and ADME studies that evaluate kinetics in one or more tumor model systems. The IND application can also be strengthened by conducting safety studies in tumor-bearing animals as a model to mimic altered homeostasis in tumor-bearing humans.

Ideally, the IND should only be filed after completion of additional preclinical studies that match the genetic heterogeneity of human tumors (multiple animal and tumor models), at least partially match tumor prone genetic profiles in a tumor-bearing host (genetically altered animals), match tissue tropism (orthotopic models), and match the most accepted

therapeutic protocols (combination therapy models). In some cases, the most appropriate models will be GEM or orthotopic models and a limited number of studies in standard mouse models are justified. Incorporation of multiple levels of efficacy testing along with careful selection of endpoints will lead to a development program that most closely matches molecular targets, therapeutic protocols and outcomes encountered in the clinic. Testing through all tiers may not be applicable or warranted and completion of all steps is not a guarantee of correlation with clinical efficacy. However, a thorough preclinical efficacy program provides greater assurance of potential therapeutic efficacy and safety than tumor modeling restricted to one or two implanted tumors in a single strain of mouse. The expense of comprehensive modeling and additional time spent in preclinical testing is modest compared to failure in phase II and phase III clinical trials.

SUMMARY

Transitions from *in vitro* to preclinical and then to clinical testing for tumor modulation remain difficult with a low rate of clinical entry for most therapeutic classes. Increased understanding of mechanisms of neoplasia through macromolecular biology, genomics and bioinformatics is helping to address treatment bottlenecks such as lack of specificity, low efficacy, toxicity and drug resistance, and helping to identify critical targets for clinical exploitation. In addition to cytotoxic, hormonal, adjunct, and medical device therapies, numerous novel strategies for enhanced and targeted drug deliver, anti-angiogenesis (inhibitors and enhanced

permeation), immunotherapy (vaccines, monoclonal antibodies, toxin conjugates, prodrug activators, cytokine antagonists), small molecules (inhibitors of growth, matrix and adhesion), apoptosis (enhancers, inducers, proteasome inhibitors, reverse DNA methylation), anti-sense and gene therapy (tumor suppressor genes) and cell cycle alterations (inhibitors) are being developed for the anti-tumor market. However, our ability to model and accurately predict clinical efficacy is limited. Despite historical significance and ongoing utility, tumor models in mice used for preclinical therapeutic intervention often error towards false positive results and curing cancer in mice. The inadequacy of classic transplantation models for anti-tumor therapy is helping to drive development and use of new models based on genetic and technical modifications. However, underlying limitations of tumor models reinforce the need for careful attention to design (applying correct models to the question), conduct (using multiple models) and interpretation (recognizing limitations and applying stringent criteria to outcomes) of efficacy studies for tumor modulation. Animal models can provide quick answers but application of these results to predicting clinical outcomes is often undertaken prematurely. New strategies and techniques, and continued improvements in stringency and consistency of criteria used for evaluating outcomes will be necessary to ensure that tumor models in mice remain a useful tool for development of anticancer agents and devices.

REFERENCES

- Abate-Shen, C., and Shen, M. M. (2002). Mouse models of prostate carcinogenesis. *Trends Genet* 18, 51-55.
- Alvarez, E. (2002). B16 murine melanoma: historical perspective on the development of a solid tumor model. *Tumor Models in Cancer Research*, Teicher B. A. Humana Press, Totowa, NJ.
- An, Z., Jiang, P., Wang, X., Moossa, A. R., and Hoffman, R. M. (1999). Development of a high metastatic orthotopic model of human renal cell carcinoma in nude mice: benefits of fragment implantation compared to cell-suspension injection. *Clin Exp Metastasis* 17, 265-70.
- Anderson, R. N. (2002). Deaths: leading causes for 2000. *National Vital Statistics Report* 50, 12-4.
- Arias, E., and Smith, B. L. (2003). Deaths: preliminary data for 2001. *National Vital Statistics Report* 51, 1-16.
- Artandi, S. E. (2002). Telomore shortening and cell fates in mouse models of neoplasia. *Trends Mol Med* 8, 44-7.
- Atassi, G., Dumont, P., Kabbe, H. J., and Yoder, O. (1988). A new antitumor agent, batracylin, selected by a preclinical solid tumor model. *Drugs Exp Clin Res* 14, 571-4.
- Averbuck, B., Schuh, J. C. L., Papay, R., and Maliszewski, C. (2002). Antitumor effects of flt3 ligand in transplanted murine tumor models. *J Immunother* 25, 27-35.
- Balmain, A. (2002). Cancer as a complex genetic trait: tumor susceptibility in humans and mouse models. *Cell* 108, 145-52.
- Bankert, R. B., Egilmez, N. K., and Hess, S. D. (2001). Human-SCID mouse chimeric models for the evaluation of anti-cancer therapies. *Trends Immunol* 22, 386-93.
- Bankert, R. B., Hess, S. D., and Egilmez, N. K. (2002). SCID mouse models to study human cancer pathogenesis and approaches to therapy: potential, limitations, and future directions. *Front Biosci* 7, 44-62.
- Bardeesy, N., Sharpless, N. E., DePablo, R. A., and Merlino, G. (2001). The genetics of pancreatic adenocarcinoma: a roadmap for a mouse model. *Semin Cancer Biol* 11, 201-8.
- Begemann, M., Fuller, G. N., and Holland, E. C. (2002). Genetic modeling of glioma formation in mice. *Brain Pathol* 12, 117-32.
- Beltlinger, C., and Debatin, K.-M. (2001). Murine models for experimental therapy of pediatric solid tumors with poor prognosis. *Int J Cancer* 92, 313-8.
- Bernardi, R., Grisendi, S., and Pandolfi, P. P. (2002). Modelling haematopoietic malignancies in the mouse and therapeutic implications. *Oncogene* 21, 3445-58.
- Berr, S. S., Roche, J. K., El-Rifai, W., Smith Jr, M. F., and Powell, S. M. (2003). Magnetic resonance imaging of gastric cancer in T1H1 knock-out mice. *Magn Reson Med* 49, 1033-6.
- Bloomston, M., Zervos, E. E., and Rosemary II, A. S. (2002). Matrix metalloproteinases and their role in pancreatic cancer: a review of preclinical studies and clinical trials. *Ann Surg Oncol* 9, 668-74.
- Bogden, A. E., Esber, H. J., Taylor, D. J., and Gray, J. H. (1974). Comparative study on the effects of surgery, chemotherapy, and immunotherapy, alone and in combination, on metastases of the 13762 mammary adenocarcinoma. *Cancer Res* 34, 1627-31.
- Boivin, G. P., Washington, K., Yang, K., Ward, J. M., Pretlow, T. P., Russell, R., Besselsen, D. G., Godfrey, V. L., Doetschman, T., Dove, W. F., Pilot, H. C., Halberg, R. B., Trzaskowiz, S. H., Groden, J., and Coffey, R. J. (2003). Pathology of mouse models of intestinal cancer: consensus report and recommendations. *Gastroenterology* 124, 762-7.
- Bonfil, R. D., Russo, D. M., Binda, M. M., Delgado, F. M., and Vinceti, M. (2002). Higher antitumor activity of vinflunine than vinorelbine against an orthotopic murine model of transitional cell carcinoma of the bladder. *Urol Oncol* 7, 159-66.
- Boone, C. W., Steele, V. E., and Kelloff, G. J. (1992). Screening for chemopreventive (anticarcinogenic) compounds in rodents. *Mutat Res* 267, 251-5.
- Boyd, D. D., Kim, S. J., Wang, H., Jones, T. R., and Gallick, G. E. (2003). A urokinase-derived peptide (A6) increases survival of mice bearing orthotopically grown prostate cancer and reduces lymph node metastasis. *Am J Pathol* 162, 619.
- Burger, M. M. (2000). UIICC study group on basic and clinical cancer research: animal models for the natural history of cancer. *Int J Cancer* 85, 303-5.
- Burgos, J. S., Rosol, M., Moats, R. A., Khanolkadyan, V., Kohn, D. B., Nelson Jr, M. D., and Laug, W. E. (2003). Time course of bioluminescent signal in orthotopic and heterotopic brain tumors in nude mice. *BioTechniques* 34, 1184-8.
- Cardiff, R. D. (2001). Validity of mouse mammary tumour models for human breast cancer: comparative pathology. *Microsc Res Tech* 52, 224-30.
- Carson III, W. E., and Walker, M. J. (2002). Animal models of melanoma. *Tumor Models in Cancer Research*, Teicher B. A. Humana Press, Totowa, NJ.
- Casas, A., Fukuda, H., Meiss, R., and Battle, A. M. (1999). Topical and intratumoral photodynamic therapy with 5-aminolevulinic acid in a subcutaneous murine mammary adenocarcinoma. *Cancer Lett* 141, 29-38.
- Castillo, R., Diaz, J. W., Veloso, A., and Gavilondo, J. (1982). Leukemoid reaction induced by different transplantable tumors in three inbred mouse strains. *Neoplasma* 29, 73-9.
- Chakrabarty, A., Hillman, G. G., Maughan, R. L., Visscher, D. W., Ali, E., Pontes, J. E., and Haas, G. P. (1994). Influence of tumor site on the therapy of murine kidney cancer. *Anticancer Res* 14, 373-8.
- Chen, J. J. W., Peck, K., Hong, T.-M., Yang, S.-C., Sher, Y.-P., Shib, J.-Y., Wu, R., Cheng, J.-L., Roffler, S. R., Wu, C.-W., and Yang, P.-C. (2001). Global analysis of gene expression in invasion by a lung cancer model. *Cancer Res* 61, 5223-30.
- Clarke, R. (2002). Human breast-cancer xenografts as models of the human disease. *Tumor Models in Cancer Research*, Teicher B. A. Humana Press, Totowa, NJ.
- Corbett, T. H., Griswold Jr, D. P., Wolpert, M. K., Venditti, J. M., and Schabel Jr, F. M. (1979). Design and evaluation of combination chemotherapy trials in experimental animal tumor systems. *Cancer Treat Rep* 63, 799-801.
- Corbett, T. H., Panchapour, C., Polin, L., Lowichik, N., Pugh, S., White, K., Kushner, J., Meyer, J., Czarniecki, J., Chinnukroh, S., Edelstein, M., LeRusso, P., Hellbrun, L., Horwitz, J. P., Grieshaber, C., Perni, R., Wentland, M., Coughlin, S., Elenbaas, S., Philbin, R., and Rake, J. (1999). Preclinical efficacy of thioxanthone SR271425 against transplanted solid tumors of mouse and human origin. *Invest New Drugs* 17, 17-27.
- Corbett, T. H., Polin, L., Roberts, B. J., Lawson, A. J., Leopold III, W. R., White, K., Kushner, J., Paluch, J., Hazeldine, S., Moore, R., Rake,

- J., and Horwitz, J. P. (2002). Transplantable syngeneic rodent tumors: solid tumors in mice. *Tumor Models in Cancer Research*, Teicher B. A. Humana Press, Totowa, NJ.
- Couse, J. F., Davis, V. L., Hanson, R. B., Jefferson, W. N., McLachlan, J. A., Bullock, B. C., Newbold, R. R., and Korach, K. S. (1997). Accelerated onset of uterine tumors in transgenic mice with aberrant expression of the estrogen receptor after neonatal exposure to diethylstilbestrol. *Mol Carcinog* 19, 236-42.
- Culp, L. A., Holleran, J. L., and Miller, C. J. (2001). Tracking prostate carcinoma micrometastasis to multiple organs using histochemical marker genes and novel cell systems. *Histol Histopathol* 16, 945-53.
- Delhian, M., Witwer, B. P., Salehi, H. A., Yuan F., and Jain, R. K. (1996). Effect of basic fibroblast growth factor, vascular endothelial growth factor/vascular permeability factor, and host microenvironment. *Am J Pathol* 149, 59-71.
- Dewhurst, M. W., Thrall, D., and MacEwen, E. G. (2002). Spontaneous pet animal cancers. *Tumor Models in Cancer Research*, Teicher B. A. Humana Press, Totowa, NJ.
- Diamant, M. J., Garcia, C., Stillitani, L., Saavedra, V. M., Manzur, T., Vauthay, L., and Klein, S. (1998). Spontaneous murine lung adenocarcinoma (P07): a new experimental model to study paraneoplastic syndromes of lung cancer. *Int J Mol Med* 2, 45-50.
- Drexler, H. G., Dirks, W. G., Matsuo, Y., and MacLeod, R. A. (2003). False leukemia-lymphoma cell lines: an update on over 500 cell lines. *Leukemia* 17, 416-26.
- Dvornik, H. F., Harvey, V. S., Estrella, P., Brown, L. J., McDonagh, J., and Dvorak, A. M. (1987). Fibrin containing gels induce angiogenesis. Implications for tumor stroma generation and wound healing. *Lab Invest* 57, 673-86.
- Dykes, D. J., and Waud, W. R. (2002). Murine L1210 and P388 Leukemias. *Tumor Models in Cancer Research*, Teicher B. A. Humana Press, Totowa, NJ.
- Eccles, S. A. (2002). Models for evaluation of targeted therapies of metastatic disease. *Tumor Models in Cancer Research*, Teicher B. A. Humana Press, Totowa, NJ.
- Edinger, M., Sweeney, T. J., Tucker, A. A., Olomu, A. B., Negrin, R. S., and Contag, C. H. (1999). Noninvasive assessment of tumor cell proliferation in animal models. *Neoplasia* 1, 303-10.
- Eto, M., Harada, M., Tamada, K., Tokuda, N., Koikawa, Y., Nakamura, M., Nomoto, K., and Naito, S. (2000). Antitumor activity of interleukin-12 against murine bladder cancer. *J Urol* 163, 1549-52.
- Fausto, N. (1999). Mouse liver tumorigenesis: models, mechanisms, and relevance to human disease. *Semin Liver Dis* 19, 243-52.
- Feitelson, M. A., and Larkin, J. D. (2001). New animal models of hepatitis B and C. *ILAR J* 42, 127-38.
- Fogh, J., Fogh, J. M., and Orfeo, T. (1977). One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *J Natl Cancer Inst* 59, 221-6.
- Fu, X., Guadagni, F., and Hoffman, R. M. (1992). A metastatic nude-mouse model of human pancreatic cancer constructed orthotopically with histologically intact patient specimens. *Proc Natl Acad Sci USA* 89, 5645-9.
- Furukawa, T., Kubota, T., Watanabe, M., Kitajima, M., and Hoffman, R. M. (1993a). A novel "patient-like" treatment model of human pancreatic cancer constructed using orthotopic transplantation of histologically intact human tumor tissue in nude mice. *Cancer Res* 53, 3070-2.
- Furukawa, T., Kubota, T., Watanabe, M., Kuo, T. H., Kitajima, M., and Hoffman, R. M. (1993b). Differential chemosensitivity of local and metastatic human gastric cancer after orthotopic transplantation of histologically intact tumor tissue in nude mice. *Int J Cancer* 54, 397-401.
- Gaudet, P., Hodgson, J. G., Eden, A., Jackson-Grusby, L., Dausman, J., Gray, J. W., Leonhardt, H., and Jaenisch, R. (2003). Induction of tumors in mice by genomic hypomethylation. *Science* 300, 489.
- Gershwin, M. E., Ikeda, R. M., Kawakami, T. G., and Owens, R. B. (1977). Immunobiology of heterotransplanted human tumors in nude mice. *J Natl Cancer Inst* 58, 1455-60.
- Giard, D. J., Aaronson, S. A., Todaro, G. J., Arminstein, P., Kersey, J. H., Dosik, H., and Parks, W. P. (1973). In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. *J Natl Cancer Inst* 51, 1417-23.
- Giovannella, B. (2002). Xenotransplantation of human cell cultures in nude mice. *Tumor Models in Cancer Research*, Teicher B. A. Humana Press, Totowa, NJ.
- Giraldi, T., Zorzet, S., Perissin, L., and Rapozzi, V. (2000). Stress and chemotherapy: combined effects on tumor progression and immunity in animal models. *Ann NY Acad Sci* 917, 549-59.
- Goodman, J. L., and Watson, R. E. (2002). Altered DNA methylation: a secondary mechanism involved in carcinogenesis. *Annu Rev Pharmacol Toxicol* 42, 501-25.
- Goytisolo, F. A., and Blasco, M. A. (2002). Many ways to telomere dysfunction: in vivo studies using mouse models. *Oncogene* 21, 584-91.
- Granger, M. P., Wright, W. E., and Shay, J. W. (2002). Telomerase in cancer and aging. *Crit Rev Oncol Hematol* 41, 29-40.
- Gunduz, N., Fisher, B., and Saffer, E. A. (1979). Effect of surgical removal on the growth and kinetics of residual tumor. *Cancer Res* 39, 3861-5.
- Gutmann, D. H., Baker, S. J., Giovannini, M., Garbow, J., and Weiss, W. (2003). Mouse models of human cancer consortium symposium on nervous system tumors. *Cancer Res* 63, 3001-4.
- Haan, B., and Balmain, A. (2001). Building 'validated' mouse models of human cancer. *Curr Opin Cell Biol* 13, 778-84.
- Harrison, S. (2002). Perspective on the history of tumor models. *Tumor Models in Cancer Research*, Teicher B. A. Humana Press, Totowa, NJ.
- Hay, R. J. (1991). Operator-induced contamination in cell culture systems. *Dev Biol Stand* 75, 193-204.
- Herber, R., Liem, A., Pilot, H., and Lambert, P. F. (1996). Squamous epithelial hyperplasia and carcinoma in mice transgenic for the human papillomavirus type 16 E7 oncogene. *J Virol* 70, 1873-81.
- Herrig, M., and Cristofari, G. (2002). Recent advances in cancer research: mouse models of tumorigenesis. *Biochim Biophys Acta* 1602, 97-113.
- Heyer, J., Yang, K., Lipkin, M., Edelman, W., and Kucherlapati, R. (1999). Mouse models for colorectal cancer. *Oncogene* 18, 5325-33.
- Hillman, G. G. (2002). Experimental animal models for renal cell carcinoma. *Tumor Models in Cancer Research*, Teicher B. A. Humana Press, Totowa, NJ.
- Hoffman, R. M. (1999). Orthotopic metastatic mouse models for anticancer drug discovery and evaluation: a bridge to the clinic. *Invest New Drugs* 17, 343-59.
- Hoffman, R. M. (2002). Green fluorescent protein imaging of tumor growth, metastasis, and angiogenesis in mouse models. *Oncology* 3, 546-56.
- Holland, E. C. (2001). Brain tumor animal models: importance and progress. *Curr Opin Oncol* 13, 143-7.
- Hoon, D. S., Fujimoto, A., Shu, S., and Taback, B. (2002). Assessment of genetic heterogeneity in tumors using laser capture microdissection. *Meth Enzymol* 356, 302-9.
- Horig, H., Wainstein, A., Long, L., Kahn, D., Soni, S., Marcus, A., Edelman, Kucherlapati, W. R., and Kaufman, H. L. (2001). A new mouse model for evaluating the immunotherapy of human colorectal cancer. *Cancer Res* 61, 8520.
- Hortz, H. G., Hines, O. J., Foitzik, T., and Reber, H. A. (2000). Animal models of exocrine pancreatic cancer. *Int J Colorectal Dis* 15, 136-43.
- Houghton, P. J., Adamson, P. C., Blaney, S., Fine, H. A., Gorlick, R., Haber, M., Helman, L., Hirschfeld, S., Hollingshead, M. G., Israel, M. A., Lock, R. B., Maris, J. M., Merlino, G., Patterson, W., Reynolds, C. P., Shannon, K., Yu, A., Yu, J., and Smith, M. A. (2002). Testing of new agents in childhood cancer preclinical models: meeting summary. *Clin Cancer Res* 8, 3646-57.
- Hursting, S. D. (1997). Experimental models of gene-environment interaction for cancer chemoprevention studies. *Curr Opin Oncol* 9, 487-91.
- Huseby, R. A. (1969). Utility and failure of models in oncology. *Fed Proc* 28, 211-5.
- Hutchinson, J. N., and Muller, W. J. (2000). Transgenic mouse models of human breast cancer. *Oncogene* 19, 6130-7.
- Jackson-Grusby, L. (2002). Modeling cancer in mice. *Oncogene* 21, 5504-14.
- Jain, R. K., Munn, J. J., and Pukamuta, D. (2002). Transparent window models and intravital microscopy: imagine gene expression, physiological function, and drug delivery in tumors. *Tumor Models in Cancer Research*, Teicher B. A. Humana Press, Totowa, NJ.

- Jonkers, J., and Berns, A. (2002). Conditional mouse models of sporadic cancer. *Nat Rev Cancer* 2, 251-65.
- Justice, M. J., Novorotsky, J. K., Weber, J. S., Zheng, B., and Bradley, A. (1999). Mouse ENU mutagenesis. *Hum Mol Genet* 8, 1955.
- Kennel, S. J., Lankford, T., Davern, S., Foote, L., Taniguchi, K., Ohizumi, I., Tsutsumi, Y., Nakagawa, S., Mayumi, T., and Mirzadeh, S. (2002). Therapy of rat tracheal carcinoma IC-12 in SCID mice: vascular targeting with [213Bi]-Mab TES-23. *Eur J Cancer* 38, 1278-87.
- Kerbel, R. S. (1999). What is the optimal rodent model for anti-tumor drug testing? *Cancer Metastasis Rev* 17, 301-4.
- Keshavavari, A., Vazey, S., Noble, M. L., Chi, E. Y., Walker, C., Martin, R. W., and Fujimoto, V. Y. (2002). Treatment of uterine leiomyosarcoma in a xenograft nude mouse model using high-intensity focused ultrasound: a potential treatment modality for recurrent pelvic disease. *Gynecol Oncol* 86, 344-50.
- Killion, J. J., Radinsky, R., and Fidler, I. J. (1999). Orthotopic models are necessary to predict therapy of transplantable tumors in mice. *Cancer Metastasis Rev* 17, 284.
- Klatt, P., and Serrano, M. (2003). Engineering cancer resistance in mice. *Carcinogenesis* 24, 817.
- Klausner, R. D. (1999). Studying cancer in the mouse. *Oncogene* 18, 5249-52.
- Knapp, D. W., and Waters, D. J. (1997). Naturally occurring cancer in pet dogs: important models for developing improved cancer therapy for humans. *Mol Med Today* 3, 8-11.
- Koback-Lanser, M., Thorup, I., Diederichsen, A., Fenger, C., and Heitinga, M. R. (2000). Review of colorectal cancer and its metastases in rodent models: comparative aspects with those in humans. *Comp Med* 50, 16-26.
- Koike, K. (2002). Hepatocarcinogenesis in hepatitis viral infection: lessons from transgenic mouse studies. *J Gastroenterol* 37, 55-64.
- Kristjansen, P. E. G. (2002). Tissue-isolated tumors in mice: ex vivo perfusion of human tumor xenografts. *Tumor Models in Cancer Research*, Teicher B. A. Humana Press, Totowa, NJ.
- Kruger, A., Schirmacher, V., and Khokha, R. (1999). The bacterial lacZ gene: an important tool for metastasis research and evaluation of new cancer therapies. *Cancer Metastasis Rev* 17, 285-94.
- Kunsfeld, R., Wickenhauser, G., Michalec, U., Teifels, M., Umek, W., Naujoks, K., Wolff, K., and Petzelbauer, P. (2003). Paclitaxel encapsulated in cationic liposomes diminishes tumor angiogenesis and melanoma growth in a "humanized" SCID mouse model. *J Invest Dermatol* 120, 476-82.
- Kuo, T.-H., Kubota, T., Watanabe, M., Furukawa, T., Kase, S., Tanino, H., Saikawa, Y., Ishibiki, K., Kitajima, M., and Hoffman, R. M. (1993). Site-specific chemosensitivity of human small-cell lung carcinoma growing orthotopically compared to subcutaneously in SCID mice: the importance of orthotopic models to obtain relevant drug evaluation data. *Anticancer Res* 13, 627-30.
- Lapidot, T., Fajerman, Y., and Kollet, O. (1997). Immune-deficient SCID and NOD/SCID mice models as functional assays for studying normal and malignant human hematopoiesis. *J Mol Med* 75, 664-73.
- Laster Jr., W. R., Schabel Jr., F. M., Skipper, H. E., Wilcox, W. S., and Thomson, J. R. (1961). Experimental evaluation of potential anticancer agents IV. host weight loss as it relates to false positives in drug evaluation. *Cancer Res* 21, 895-906.
- Li, C. Y., Shan, S., Huang, Q., Braun, R. D., Lanzan, J., Ito, K., Lin, P., and Dewhirst, M. W. (2000). Initial stages of tumor cell-induced angiogenesis: evaluation via skin window chambers in rodent models. *J Natl Cancer Inst* 92, 143-7.
- Liebelt, R. A., Gehring, G., Delmonte, L., Schuster, G., and Liebelt, A. G. (1974). Paraneoplastic syndromes in experimental animal model systems. *Ann NY Acad Sci* 230, 547-64.
- Lin, J., Sahakian, D. C., de Moraes, S. M., Xu, J. J., Polzer, R. J., and Winter, S. M. (2003). The role of absorption, distribution, metabolism, excretion and toxicity in drug discovery. *Curr Top Med Chem* 3, 1125-4.
- Liu, J., and Johnston, M. R. (2002). Animal models for studying lung cancer and evaluating novel intervention strategies. *Surg Oncol* 11, 217-27.
- Maicherhofer, C., Gangnus, R., Diebold, J., and Speicher, M. R. (2003). Multicolor deconvolution microscopy of thick biological specimens. *Am J Pathol* 162, 373.
- Malkinson, A. M. (2001). Primary lung tumors in mice as an aid for understanding, preventing, and treating human adenocarcinoma of the lung. *Lung Cancer* 32, 265-79.
- Manivasagar, V., Heng, P. W., Hiao, J., Zheng, W., Soo, K. C., and Olivo, M. (2002). Macro-microscopic fluorescence imaging of human NPC xenografts in a murine model using topical vs intravenous administration of 5-aminolevulinic acid. *Int J Oncol* 21, 1003-7.
- Manzotti, C., Andisio, R. A., and Pratesi, G. (1993). Importance of orthotopic implantation for human tumors as model systems: relevance to metastasis and invasion. *Clin Exp Metastasis* 11, 5-14.
- McClatchey A. I. (1999). Modeling metastasis in the mouse. *Oncogene* 18, 5334-9.
- Menon, K., and Teicher, B. A. (2002). Metastasis models: lungs, spleen/liver, bone, and brain. *Tumor Models in Cancer Research*, Teicher B. A. Humana Press, Totowa, NJ.
- Mewissen, R., Jonkers, J., and Berns, A. (2001). Mouse models for sporadic cancer. *Exp Cell Res* 264, 100-10.
- Mickisch, G. H., Merlino, G. T., Galski, H., Gottesman, M. M., and Pastan, I. (1991). Transgenic mice that express the human multidrug-resistance gene in bone marrow enable a rapid identification of agents that reverse drug resistance. *Proc Natl Acad Sci USA* 88, 547-51.
- Monks, W. L., Carreira, C. M., Tszuk, Y., Gohongi, T., Fukumura, D., and Jain, R. K. (2002). Role of host microenvironment in angiogenesis and tumor vascular functions in human breast cancer xenografts: mammary fat pad versus cranial tumors. *Clin Cancer Res* 8, 1008-13.
- Moore, K. J., and Nagle, D. L. (2000). Complex trait analysis in the mouse: the strengths, the limitations and the promise yet to come. *Annu Rev Genet* 34, 653-86.
- Mosley, G. W., Elliott, J., Wright, M. D., Partridge, L. J., and Monk, P. N. (2003). Interspecies contamination of the KM3 cell line: implications for CD63 function in melanoma metastasis. *Int J Cancer* 105, 613-6.
- Mukherjee, P., El-Abadi, M. M., Kasperczyk, J. L., Rames, M. K., and Seyfried, T. N. (2002). Dietary restriction reduced angiogenesis and growth in an orthotopic mouse brain tumor model. *Br J Cancer* 86, 1615-21.
- Myers, J. N., Holsinger, F. C., Jasser, S. A., Bekele, B. N., and Fidler, I. J. (2002). An orthotopic nude mouse model of oral tongue squamous cell carcinoma. *Clin Cancer Res* 8, 293-8.
- Naf, D., Krupke, D. M., Sundberg, J. P., Eppig, J. T., and Bult, C. J. (2002). The mouse tumor biology database: a public resource for cancer genetics and pathology of the mouse. *Cancer Res* 62, 1235.
- Naito, S., Giavazzi, R., Walker, S. M., Itoh, K., Mayo, J., and Fidler, I. J. (1987a). Growth and metastatic behavior of human tumor cells implanted into nude and beige nude mice. *Clin Exp Metastasis* 5, 135-46.
- Naito, S., von Eschenbach, A. C., and Fidler, I. J. (1987b). Different growth pattern and biologic behavior of human renal cell carcinoma implanted into different organs of nude mice. *J Natl Cancer Inst* 78, 377-85.
- Navone, N. M., Logothetis, C. J., von Eschenbach, A. C., and Troncoso, P. (1999). Mouse models of prostate cancer: uses and limitations. *Cancer Metastasis Rev* 17, 361-71.
- Nelson, A. L., Algon, S. A., Munasinghe, J., Graves, O., Goumnerova, L., Burstein, D., Pomeroy, S. L., and Kim, J. Y. (2003). Magnetic resonance imaging of patched heterozygous and xenografted mouse brain tumors. *J Neurooncol* 62, 259-67.
- Nicklas, W., Kraft, V., and Meyer, B. (1993). Contamination of transplantable tumors, cell lines, and monoclonal antibodies with rodent viruses. *Lab Anim Sci* 43, 296-300.
- Nyska, A., Dayan, A., and Maronit, R. R. (2002). New tools in therapeutic research—prostatic cancer and models. *Toxicol Pathol* 30, 283-7.
- Opitz, O. G., Harada, H., Suliman, Y., Rhoades, B., Sharpless, N. E., Kent, R., Kopolovich, L., Nakagawa, H., and Rustgi, A. K. (2002). A mouse model of human oral-esophageal cancer. *J Clin Invest* 110, 761.
- Orsulic, S., Li, Y., Soslow, R. A., Vitale-Cross, L. A., Gutkind, J. S., and Varmus, H. E. (2002). Induction of ovarian cancer by defined multiple genetic changes in a mouse model system. *Cancer Cell* 1, 53-62.
- Paddock, S. W. (1999). Confocal laser scanning microscopy. *BioTechniques* 27, 992-1004.

- Pettaway, C. A., Pathak, S., Greene, G., Ramirez, E., Wilson, M. R., Killian, J. J., and Fidler, I. J. (1996). Selection of highly metastatic variants of different human prostatic carcinomas using orthotopic implantation in nude mice. *Clin Cancer Res* 2, 3627-36.
- Pitot, H. C. (2001). Animal models of neoplastic development. *Develop Biol* 106, 53-7.
- Rahman, N. A., and Huhtaniemi, I. T. (2001). Ovarian tumorigenesis in mice transgenic for murine inhibin alpha subunit promoter-driven Simian Virus 40 T-antigen oncotag, functional characteristics, and endocrine effects. *Biol Reprod* 64, 1122-30.
- Rahman, N. A., Kananen Riihanen, K., Paukku, T., Mikola, M., Markkula, M., Hämäläinen, T., and Huhtaniemi, I. T. (1998). Transgenic mouse models for gonadal tumorigenesis. *Mol Cell Endocrinol* 145, 167-74.
- Ray, P., Wu, A. M., and Gambhir, S. S. (2003). Optical bioluminescence and positron emission tomography imaging of a novel fusion reporter gene in tumor xenografts of living mice. *Cancer Res* 63, 1160-5.
- Reilly, K. M., and Jacks, T. (2001). Genetically engineered mouse models of astrocytoma: CEMs in the rough? *Semin Cancer Biol* 11, 177-91.
- Resor, L., Bowen, T. J., and Wynshaw-Boris, A. (2001). Unraveling human cancer in the mouse: recent refinements to modeling and analysis. *Hum Mol Genet* 10, 669-75.
- Rew, D. A. (2000a). Modelling in tumour biology part I: modelling concepts and structures. *Eur J Surg Oncol* 26, 78-94.
- Rew, D. A. (2000b). Modelling in tumour biology part II: modelling cancer therapy. *Eur J Surg Oncol* 26, 181-8.
- Ridout III, W. M., Wakayama, T., Wutz, A., Eggan, K., Jackson-Grusby, L., Dausman, J., Yangimichal, R., and Jaenisch, R. (2000). Generation of mice from wild-type and targeted ES cells by nuclear cloning. *Nat Genet* 24, 109-10.
- Righi, L., Deaglio, S., Pecchioni, C., Gregorini, A., Hornstein, A. L., Bussolati, G., Sapino, A., and Malavasi, F. (2003). Role of CD31/platelet endothelial cell adhesion molecule-1 expression in vitro and in vivo growth and differentiation of human breast cancer cells. *Am J Pathol* 162, 1163.
- Rosenberg, E., Holmes, M., Tenenholz, T., Khalil, A. E. R., and Valerie, K. (2003). Preservation of fluorescent protein activity in tumor tissue. *BioTechniques* 34, 476-80.
- Rosenberg, M. P., and Bortner, D. (1999). Why transgenic and knockout animal models should be used (for drug efficacy studies in cancer). *Cancer Metastasis Rev* 17, 295-9.
- Rosner, A., Miyoshi, K., Landesman-Bollag, E., Xu, X., Seldin, D. C., Moser, A. R., MacLeod, C. L., Shyamala, G., Gillgrass, A. E., and Cardiff, R. D. (2002). Pathway pathology: histological differences between ErbB/Ras and wnt pathway transgenic mammary tumors. *Am J Pathol* 161, 1087.
- Royal, R., Lange, P. H., and Vessella, R. (1996). Preclinical models of prostate cancer. *Semin Oncol* 23, 35-40.
- Ruggero, D., Grisendi, S., Piazza, F., Rego, E., Mari, F., Rao, P. H., Cordon-Cardo, C., and Pandolfi, P. P. (2003). Dyskeratosis congenita and cancer in mice deficient in ribosomal RNA modification. *Science* 299, 259.
- Sadar, M. D., Akopian, V. A., and Beraldi, E. (2002). Characterization of a new in vivo hollow fiber model for the study of progression of prostate cancer to androgen independence. *Mol Cancer Ther* 1, 629-37.
- Schipper, H., Goh, C. R., and Wang, T. L. (1995). Shifting the cancer paradigm: must we kill to cure? *J Clin Oncol* 801-7.
- Schwartz, J., and Gu, X. (2002). Hamster oral cancer model. *Tumor Models in Cancer Research*, Teicher B. A., Humana Press, Totowa, NJ.
- Seki, N., Hayakawa, Y., Brooks, A. D., Wine, J., Willrott, R. H., Yagita, H., Tancer, J. E., Smyth M. J., and Sayers, T. J. (2003). Tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis is an important endogenous mechanism for resistance to liver metastases in murine renal cancer. *Cancer Res* 63, 207-13.
- Sharma, P., and Schreiber-Agus, N. (1999). Mouse models of prostate cancer. *Oncogene* 18, 5349-55.
- Siemann, D. W. (1987). Satisfactory and unsatisfactory tumor models factors influencing the selection of a tumor model for experimental evaluation. *Rodent Tumor Models in Experimental Cancer Therapy*, Kallman, R. F., Pergamon Books, Inc., Elmsford, NY.
- Skipper, H. E. (1968). Biochemical, biological, pharmacologic, toxicologic, kinetic, and clinical (subhuman and human) relationships. *Cancer* 21, 600-10.
- Skipper, H. E. (1990). Tumor differences, drug differences, treatment design differences, and effects on therapeutic outcome (degree and duration of therapeutic response). *Prog Clin Biol Res* 354, 81-98.
- Spitsbergen, J. M., and Kent, M. L. (2003). The state of the art of the zebrafish model for toxicology and toxicologic pathology research—advantages and current limitations. *Toxicol Pathol* 31, 62-87.
- Standop, J., Schneider, M. B., Ulrich, A., and Pour, P. M. (2001). Experimental animal models in pancreatic carcinogenesis: lessons for human pancreatic cancer. *Dis Dig* 19, 24-31.
- Steel, G. G. (1987). How well do xenografts maintain the therapeutic response characteristics of the source tumor in the donor patient? *Rodent Tumor Models in Experimental Cancer Therapy*, Kallman, R. F., Pergamon Books, Inc., Elmsford, NY.
- Strandberg, J. D. (2000). Comparative pathology of benign prostatic hyperplasia. *Prostatic Diseases*, Lepor, H. W. Saunders Co., Philadelphia, PA.
- Suttio, A., Nyska, A., Haseman, J. K., Moser, G. J., Hackett, T. R., and Goldworthy, T. L. (2003). A grading scheme for the assessment of proliferative lesions of the mouse prostate in the TRAMP model. *Toxicol Pathol* 31, 31-38.
- Tanaka, R., Okada, M., Kajimura, N., Otsubo, K., Oyokuni, N., Nagasaki, K., Kimoto, H., and Yamaguchi, K. (1996). Triple paraneoplastic syndrome of hypercalcemia, leukocytosis and cachexia in two human tumor xenografts in nude mice. *Jpn J Clin Oncol* 26, 88-94.
- Teicher, B. A. (1997). Models for minimal residual tumor. *Anticancer Drug Development Guide: Preclinical Screening, Clinical Trials, and Approval*. Teicher, B. A. Humana Press Inc., Totowa, NJ.
- Teicher, B. A. (2002). In vivo tumor response end points. *Tumor Models in Cancer Research*, Teicher, B. A., Humana Press, Totowa, NJ.
- Thompson, H. J., and Sporn, M. B. (2002). Mammary cancer in rats. *Tumor Models in Cancer Research*, Teicher, B. A., Humana Press, Totowa, NJ.
- Trainer, D. L., Kline, T., McCabe, F. L., Faucette, L. F., Feld, J., Chaikin, M., Anzano, M., Riemann, D., Hoffstein, S., Li, D.-J., Gonnardo, D., Buscarino, C., Lynch, M., Poste, G., and Greig, R. (1988). Biological characterization and oncogene expression in human colorectal carcinoma cell lines. *Int J Cancer* 41, 287-96.
- Tuveson, D. A., and Jacks, T. (1999). Modeling human lung cancer in mice: similarities and shortcomings. *Oncogene* 18, 5318-24.
- Tuveson, D. A., and Jacks, T. (2002). Technologically advanced cancer modeling in mice. *Curr Opin Genet Dev* 12, 105-10.
- Uckun, F. M., and Senesi, M. G. (2002). SCID mouse models of human leukemia and lymphoma as tools for new agent development. *Tumor Models in Cancer Research*, Teicher B. A., Humana Press, Totowa, NJ.
- UKCCCR. (1998). United Kingdom coordinating committee on cancer research (UKCCCR) guidelines for the welfare of animals in experimental neoplasia (second edition). *Br J Cancer* 77, 1-10.
- Vail, D. M., and MacEwen, E. G. (1997). Spontaneously occurring tumors in companion animals as models for drug development. *Anticancer Drug Development Guide: Preclinical Screening, Clinical Trials, and Approval*, Teicher, B. A., Humana Press, Totowa, NJ.
- van Dyke, T., and Jacks, T. (2002). Cancer modeling in the modern era: progress and challenges. *Cell* 108, 135-44.
- Vanchieri, C. (2001). Move over, mouse: make way for the woodchucks, ferrets, and zebrafish. *J Natl Cancer Inst* 93, 418-9.
- Vanderkerken, K., De Leener, E., Shipman, C., Asohara, K., Willems, A., van Camp, B., and Croucher, P. (2003). Recombinant osteopontin decreases tumor burden and increases survival in a murine model of multiple myeloma. *Cancer Res* 63, 287-9.
- Wallace, J. (2000). Humane endpoints and cancer research. *ILAR J* 41, 87-93.
- Waters, D. J., and Bostwick, D. G. (1997). The canine prostate is a spontaneous model of intracapsular neoplasia and prostate cancer progression. *Anticancer Res* 17, 1467-70.
- Waters, D. J., Bostwick, D. G., and Murphy, G. P. (1998). Conference summary: first international workshop on models of prostate cancer. *Prostate* 36, 47-48.

- Watson, A., Wu, X., and Bruchez, M. (2003). Lighting up cells with quantum dots. *BioTechniques* 34, 296-303.
- Wetterwald, A., van der Pluijm, G., Que, I., Sijmons, B., Buijs, J., Karperien, M., Lowik, C. W. G. M., Gautschi, E., Thalmann, G. N., and Cecchini, M. G. (2002). Optical imaging of cancer metastasis to bone marrow: a mouse model of minimal residual disease. *Am J Pathol* 160, 1143.
- Wheeler, D. L., Church, D. M., Federhen, S., Lash, A. E., Madden, T. L., Pontius, J. U., Schuler, G. D., Schriml, L. M., Sequeira, E., Tatusova, T. A., and Wagner, L. (2003). Database resources of the national center for biotechnology. *Nucleic Acids Res* 31, 28-33.
- Wichmann, M. W., Muller, C., Meyer, G., Adam, M., Angele, M. K., Eisenmenger S. J., and Schildberg, F. W. (2003). Different immune responses to abdominal surgery in men and women. *Langenbeck's Arch Surg* 387, 397-401.
- Wilmanns, C., Fan, D., O'Brian, C. A., Bucana, C. D., and Fidler, I. J. (1992). Orthotopic and ectopic organ environments differently influence the sensitivity of murine colon carcinoma cells to doxorubicin and 5-fluorouracil. *Int J Cancer* 52, 78-104.
- Yang, H., Berger, F., Tran, C., Gambhir, S. S., and Sawyers, C. L. (2003). MicroPET imaging of prostate cancer in LNCAP-SR39TK-GFP mouse xenografts. *Prostate* 55, 39-47.
- Yoneda, T., Aufdemorte, T. B., Nishimura, R., Nishikawa, N., Sakuda, M., Alsina, M. M., Chavez, J. B., and Mundy, G. R. (1991). Occurrence of hypercalcemia and leukocytosis with cachexia in a human squamous cell carcinoma of the maxilla in athymic nude mice: a novel experimental model of three concomitant paraneoplastic syndromes. *J Clin Oncol* 9, 468-77.
- Zan, Y., Haag, J. D., Chen, K. S., Shepel, L. A., Wigington, D., Wang, Y. R., Hu, R., Lopez-Guajardo, C. C., Brose, H. L., Porter, K. I., Leonard, R. A., Hiit, A. A., Schommer, S. L., Elegbede, A. F., and Gould, M. N. (2003). Production of knockout rats using ENU mutagenesis and a yeast-based screening assay. *Nat Biotechnol* 21, 645-51.
- Zhang, Z., Yin, L., Zhang, Y., and Zhao, F. (2002). In situ transduction of cytosine deaminase gene followed by systemic use of 5-fluorocytosine inhibits tumor growth and metastasis in orthotopic prostate cancer mouse models. *Chin Med J* 115, 227-31.

National
Library
of MedicineMy NC
[Sign In] [Regs]

All Databases PubMed Nucleotide Protein Genome Structure OMIM PMC Journals Bool

Search PubMed for

Limits Preview/Index History Clipboard Details
Display Abstract Show 20 Sort by Send to

About Entrez

Text Version

All: 1 Review: 0

Entrez PubMed

Overview
Help | FAQ
Tutorial
New/Noteworthy
E-Utilities

1: Cell. 1977 Jun;11(2):273-81.

Related Articles, Links

**Analysis of VSV mutant with attenuated cytopathogenicity:
mutation in viral function, P, for inhibition of protein synthesis.****Stanners CP, Francoeur AM, Lam T.**PubMed Services
Journals Database
MeSH Database
Single Citation Matcher
Batch Citation Matcher
Clinical Queries
Special Queries
LinkOut
My NCBI (Cubby)Related Resources
Order Documents
NLM Catalog
NLM Gateway
TOXNET
Consumer Health
Clinical Alerts
ClinicalTrials.gov
PubMed Central

T1026, a ts mutant of VSV which is much less cytopathogenic than its parent, HR, and which can establish persistent infection under certain conditions, is a double mutant. In addition to its ts mutation in the virion RNA polymerase, T1026 has a second non-ts mutation in a viral function termed "P". This function is responsible for the inhibition of total protein synthesis in infected cells and acts chiefly at the level of translational initiation. In some cell systems, the inhibition of protein synthesis produced by P appears to be selective for cellular protein synthesis, whereas in other cell systems, both cellular and viral protein synthesis are inhibited. T1026 and its ts revertants are phenotypically P- -that is, cells infected with them show total protein synthesis rates equal to or greater than uninfected cells, while synthesizing viral proteins at the same or even greater rates than HR-infected cells. The P- mutation is correlated with failure to increase plaque size after 2-3 days of incubation. Since viral mutants obtained from persistently infected cultures in a variety of systems appear to be double mutants with a ts mutation in the virion RNA polymerase and a small plaque marker, we suggest that T1026 could represent a model for such mutants.

PMID: 196757 [PubMed - indexed for MEDLINE]

Display Abstract Show 20 Sort by Send to

Write to the Help Desk
NCBI | NLM | NIH
Department of Health & Human Services
Privacy Statement | Freedom of Information Act | Disclaimer

Jun 27 2005 04:57:30

Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus

DAVID F. STOJIL^{1,2}, BRIAN LICHTY¹, SHANE KNOWLES¹, RICARDO MARIUS¹, HAROLD ATKINS¹,
NAHUM SONENBERG³ & JOHN C. BELL^{1,2†}

Ottawa Regional Cancer Centre Research Laboratories, 501, Smyth Road, Ottawa, ON, Canada K1H

8L6/Department of Biochemistry, Microbiology and Immunology, University of Ottawa, 451,

Smyth Road, Ottawa, ON, Canada K1H 8M5

³Department of Biochemistry, McGill University, 3655, Drummond Street, Montreal, PQ, Canada, H3G 1Y6

D.F.S. and B.L. contributed equally to this article.

Correspondence should be addressed to J.C.B.; email: jbell@med.ottawa.ca

Interferons are circulating factors that bind to cell surface receptors, activating a signaling cascade, ultimately leading to both an antiviral response and an induction of growth inhibitory and/or apoptotic signals in normal and tumor cells¹. Attempts to exploit the ability of interferons to limit the growth of tumors in patients has met with limited results² because of cancer-specific mutations of gene products in the interferon pathway^{3,4}. Although interferon-non-responsive cancer cells may have acquired a growth/survival advantage over their normal counterparts, they may have simultaneously compromised their antiviral response. To test this, we used vesicular stomatitis virus (VSV), an enveloped, negative-sense RNA virus⁵ exquisitely sensitive to treatment with interferon⁶. VSV rapidly replicated in and selectively killed a variety of human tumor cell lines even in the presence of doses of interferon that completely protected normal human primary cell cultures. A single intratumoral injection of VSV was effective in reducing the tumor burden of nude mice bearing subcutaneous human melanoma xenografts. Our results support the use of VSV as a replication-competent oncolytic virus and demonstrate a new strategy for the treatment of interferon non-responsive tumors.

production of virus particles but also in the cytopathic effect seen at the microscopic level. At 12 hours after infection, SK-MEL melanoma cell cultures showed extensive cytopathic effect and were completely obliterated by 24 hours (Fig. 1). Pretreatment with interferon failed to protect melanoma cells from being killed by VSV. Primary human fibroblasts (AG1522) and epithelial cells (prostate epithelial cells and human ovarian surface epithelial cells) showed no overt cytopathic effect at 12 or 24 hours after infection (data not shown), but by 36 hours after infection they began to show signs of the cytopathic effect associated with VSV replication (Fig. 1). In contrast to the melanoma cultures, however, pretreatment of normal primary cells with interferon was protective and rendered them indistinguishable from uninfected cells even at 72 hours after infection (Fig. 1). We obtained similar results for all of the tumor and normal cells in Table 1 (data not shown). In these experiments, target cells were infected at a multiplicity of infection (MOI) of 0.1 plaque-forming units (PFU) per cell. Thus, the extensive cytopathic effect in the tumor cell population reflects the rapid growth and spread of VSV through the tumor cell culture. We consistently found a more rapid and fulminant growth of VSV in tumor cells than in primary normal cell cultures of fibroblasts.

We infected normal and tumor cell lines with Indiana strain of vesicular stomatitis virus (VSV) in the presence and absence of interferon. Infection of primary human fibroblasts (OSF 7, 12 and 16), primary human prostate epithelial cells and primary human ovarian surface epithelial cells produced lower virus yields after an overnight infection than did parallel infections of a range of human tumor cells (Table 1). Moreover, pre-treatment of the normal cell cultures with interferon reduced viral production to less than 1,000 infectious viral particles per ml, whereas tumor cell lines continued to produce copious amounts of virus particles (10^5 – 10^6 plaque-forming units per ml). Some tumor cell lines (such as C13) had some reduction in virus yield after interferon priming, indicating that they have an impaired but not completely defective interferon response. The differences between the various cell types was reflected not only in the

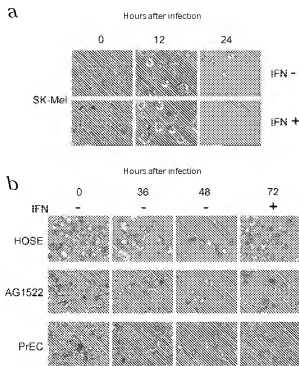
Table 1 Virus yield after overnight infection of cell lines either untreated or treated with interferon

Cell line	Viral titer (FFU/ml)	
	Untreated	Interferon- α
OSF7 (primary normal human fibroblast)	1×10^6	<10
OSF12 (primary normal human fibroblast)	2×10^6	<10
OSF16 (primary normal human fibroblast)	1×10^6	<10
REC (primary normal human prostate epithelium)	8×10^5	<10
HOSE (primary normal human ovarian surface epithelium)	1×10^5	<1,000
A2780 (human ovarian carcinoma)	2×10^6	1×10^5
OVCA 420 (human ovarian carcinoma)	1×10^6	3×10^5
C13 (human ovarian carcinoma)	1×10^6	1×10^5
LC80 (human lung carcinoma)	2×10^6	6×10^5
SK-MEL3 (human melanoma)	1×10^6	1×10^5
LNCAP (human prostate carcinoma)	4×10^6	5×10^5
HCT116 (human colon carcinoma)	1×10^6	2×10^5
293T (HEK cells transformed with T antigen and Ad virus E1A)	1×10^6	8×10^5

Normal and transformed cell lines were either untreated or pre-treated with 100 units of interferon- α , infected at an MOI of 0.1 FFU/ml and incubated for 18 h at 37 °C. VSV production titers were determined in culture medium from each sample. Ad, adenovirus.

ARTICLES

Fig. 1 The VSV-induced cytopathic effect is visible in human melanoma cells but not in primary human cells with or without interferon- α . Gelatin-coated coverslips with normal human cells and SK-MEL3 cells untreated or pretreated with 100 U/ml interferon- α were infected with VSV at an MOI of 0.1 PFU/ml. **a**, The human melanoma cells (SK-MEL3) shows cytopathic effect at 12 h after infection even in the presence of interferon. At 24 h after infection, these malignant cells have died and lifted from the coverslip. **b**, Human primary cells, including foreskin fibroblasts (AG1522), ovarian surface epithelial cells (HOSE) and prostate epithelial cells (PrEC), do not show a cytopathic effect until 36 h in the absence of interferon, and are completely protected in the presence of interferon beyond 72 h after infection.



tic or epithelial origin. Although interferon is effective in protecting normal cells from killing by VSV, it is unable to do so in a broad range of tumor types.

We tested the ability of VSV to be a selective oncolytic agent. In this experiment, we used the human 293T transformed cell line that expresses simian virus 40 large T antigen, and normal human foreskin fibroblasts. Using antibodies against large T antigen we were able to distinguish 293T cells (Fig. 2, red and blue staining) from human foreskin fibroblasts (Fig. 2, blue staining). We cultured equal numbers of each cell type on coverslips and infected them at an MOI of 0.1 PFU/cell in the presence and absence of interferon. Initially, both cell types had a spindle-like morphology with large oval nuclei. After 12 hours, most of the 293T cells showed nuclear changes (data not shown) and by 24 hours after infection, all of the remaining cells expressing large T antigen had very condensed or fragmented nuclei reminiscent of apoptosis (Fig. 2) even in the presence of interferon. As expected, the human foreskin fibroblasts were protected from VSV beyond 24 hours in the absence of interferon and beyond 36 hours in the presence of interferon (Fig. 2). This experiment demonstrates the highly selective destruction of malignant cells by VSV, a phenomenon made even more salient by interferon administration.

An essential, dose-limiting site of conventional cancer therapies is the bone marrow. We found, however, that bone marrow cultures from two separate healthy donors produced no infectious VSV particles, even when infected at an MOI of 10 PFU/cell. Moreover, the infected bone marrow cultures were indistinguishable from mock-infected (medium alone) cultures in their ability to form the normal spectrum of hematopoietic cell types after *in vitro* culture in methylcellulose. In contrast, acute myelogenous leukemia (AML) cell lines OCI/AML3, OCI/AML4 and OCI/AML5 were very susceptible to VSV infection, with 0.05 PFU/cell killing 50% of the cells at 24 hours and

as little as 0.0003 PFU/cell killing 50% at 48 hours. In co-cultures of leukemic OCI/AML3 cells mixed with normal bone marrow cells (at a ratio of 1:9), VSV again had selective oncolytic properties. In this experiment (Table 2), co-cultures were infected with VSV at an MOI of 1 PFU/cell or 5 PFU/cell for 24 hours and then were plated in methylcellulose with or without growth factors. In the presence of growth factors, both normal marrow and tumor cells will grow, whereas only OCI/AML3 cells can form colonies in the absence of growth factors. We counted colonies after 14 days and found complete ablation of growth-factor-independent leukemic cells and sparing of normal bone marrow progenitors (Table 2). We obtained identical results using a 1:3 mixture of OCI/AML3 cells and normal marrow (data not shown). These data show the selective destruction of leukemic cells in a mixed population of normal marrow and, with the data above, advocate the potential utility of VSV in *ex vivo* bone marrow purging.

Human tumor xenografts in nude mice provide a convenient model for studying potential anti-cancer therapeutics, including certain oncolytic viruses¹⁰. For VSV, however, this system is somewhat limited, as the immunological impairment of this mouse strain ultimately renders it susceptible to killing by VSV (refs. 11,12). The rapid killing of tumor cells with VSV indicated that it might be possible, however, to assess the *in vivo* oncolytic properties of the virus in an acute short term experiment. Thus, we implanted SK-MEL3 cells subcutaneously on both flanks of BALB/c nude mice and allowed these to form palpable tumors before directly infecting them with VSV. Given the recent demonstration of improved delivery of herpes simplex virus in the treatment of ovarian cancer using virus-producing cells rather than virus alone¹³, we also tested VSV-producing cells in this melanoma xenograft model. We assessed the size of the tumors and the well-being of the mice daily. Tumors not injected with VSV showed unabated growth (Fig. 3a). In contrast, tumors injected with a single dose of VSV

Table 2 Selective killing of AML cells co-cultured with normal bone marrow

Colony Type	Multiplicity of infection		
	0.0	1.0	5.0
Leukemic	172	0*	0*
Neutrophil	12	7	5
Mixed	6	3	4
Monocyte	10	7	5

The growth-factor-independent cell line OCI/AML3 was mixed at a ratio of 1:9 with normal bone marrow and infected for 24 h with VSV. Various dilutions of cells were then plated in methylcellulose with and without growth factors, and colonies were counted 14 d later. Data represent dishes receiving 10^5 cells \times . No leukemic colonies were detected on the dishes lacking growth factor even when 10^5 cells were plated per dish.

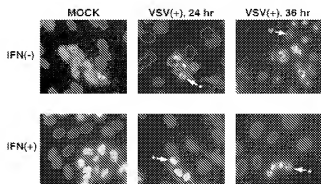


Fig. 2 VSV selectively kills transformed cells co-cultured with normal fibroblasts. Equal numbers of 293T cells and normal human fibroblasts were plated on gelatin-coated coverslips and infected at an MOI of 0.1 in the presence (+) and absence (-) of interferon. Cells were fixed at 12 h (not shown), 24 h and 36 h after infection. Fixed cells were stained with an antibody against large T antigen and DAPI. The red-staining 293T cells are killed as early as 12 h after infection, regardless of interferon treatment, with the few remaining cells showing condensed or fragmented nuclei (*). The normal fibroblasts showed altered nuclei (ψ) by 36 h after infection in the absence of interferon but are protected from the virus in the presence of interferon beyond this time. MOCK no virus (medium only).

or cells producing VSV stopped growing and in some cases decreased in size (Fig. 3a). In these experiments, BALB/c nude mice began to show symptoms of VSV infection and began dying at day 6, as in earlier studies with this strain^{11,12}. In our experience, CD-1 mice are more tolerant to VSV infection than are mice with a BALB/c genetic background, and for this reason we repeated the experiment using CD-1-derived nude mice bearing human melanoma xenografts, as described above (Fig. 3b). In addition, we attempted to rescue mice from VSV-induced mortality by treating a subset of these mice with

interferon. A single intratumoral injection of live VSV was tumor static in all cases (Fig. 3b). All tumors demonstrated at least a partial regression, with complete regressions in three of the twelve mice treated with live virus. In contrast, tumors treated with VSV inactivated by ultraviolet irradiation grew unabated until day 11 after infection, at which time the mice were killed because of severe tumor burden (Fig. 3b). Although all mice survived longer than in our initial experiment (Fig. 3), mice not receiving interferon began to die by day 10 after infection, with two of six remaining healthy for the duration of the experiment. However, all interferon-treated mice were protected and survived symptom-free for more than 45 days (four of four mice).

Over the last several decades there have been many reports correlating virus infections with tumor regression^{14,16}. With an increasing understanding of the molecular events leading to the generation and evolution of malignancies, it is now possible to begin tailoring and/or selecting viruses for their ability to replicate preferentially in tumor cells. The Onyx-015 adenovirus mutant is believed to have enhanced replication in tumor cells lacking a functional p53 protein, although reports indicate that other cellular gene products could be influencing the growth of this virus¹⁷. Another promising approach is to alter virus tropism by modifying viral surface antigens or by conditionally expressing toxic gene products with tissue-specific gene promoter elements. Alternatively, as we suggest here, the ability of a virus to selectively kill tumor cells may be determined by cancer-specific defects in innate anti-viral responses. Given our results here and other published studies, defects or downregulation of components of the interferon pathway may be a common feature of a broad range of malignancies^{5,7,18,19}, and these defects may be responsible for the tumor specificity demonstrated by a variety of oncolytic viruses^{20,19,16}.

Interferon induced by VSV infection may protect normal tissues from viral killing, whereas tumor cells, which have lost their

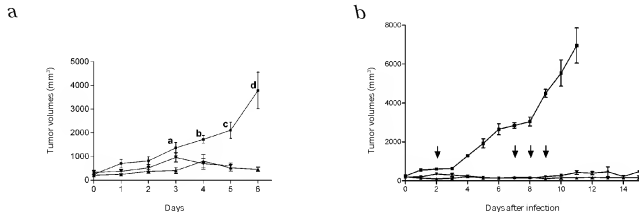


Fig. 3 VSV inhibits growth of human melanoma xenografts in nude mice. **a**, SK-MEL-3-derived tumors were developed in female BALB/c athymic mice 8–10 weeks old. On day 0, tumors were either left untreated (●) or were infected with 10^6 PFU VSV in culture medium (▼) or SK-MEL-3 cells infected with 2.5×10^6 VS (VSV-producing cells, ▲). Tumors were measured daily for 6 d, at which time infected mice were killed because of complications associated with VSV infection of nude mice. *, $P < 0.001$, on day 3, only tumors treated with VSV producing cells are significantly smaller than untreated tumors; **, $P < 0.01$; ***, $P < 0.001$.

0.001; ****, $P = 0.007$; treated compared with untreated. There were no statistically significant differences in tumor volumes between groups, days 0–2. Data represent means \pm s.e.m. from multiple tumors untreated, $n = 8$; VSV-producing cells, $n = 8$; VSV alone, $n = 4$. **b**, SK-MEL-3-derived tumors were developed in CD-1 nude mice as described above. On day 0, tumors were infected with 1×10^6 PFU live VSV (▼) or VSV inactivated by ultraviolet irradiation (■), and were measured daily. Interferon was administered to a subset of mice (▲, VSV IFN; times ►). UV-VSV, $n = 4$; VSV IFN, $n = 6$; VSV, $n = 6$.

interferon responsiveness, become selective targets for the virus. VSV replicates in tumor cells, leading to cytolysis and spread of the virus to neighboring tumor cells, whereas the surrounding normal tissue is rendered resistant to infection through the activation of various interferon-mediated defenses (such as RNA dependent protein kinase, inducible nitric oxide synthase, 2' 5' oligoadenylate synthetase and so on). Perhaps most importantly, whereas growth of VSV in a wide range of tumor cells is refractory or only moderately affected by interferon treatment, replication in normal cells *in vitro* and *in vivo* can be controlled by exogenous administration of interferon, ultimately leading to an increase in the therapeutic index of this anti-tumor therapy. Some malignant cells retain an intact interferon response, as this cytokine has been used to inhibit the growth of some cancers; however, the benefits of interferon are often short-lived. During cancer progression, as interferon-resistant tumor cells arise, they would become targets for VSV therapy.

Here we investigated the use of VSV as an oncolytic agent. VSV is readily grown to high titers, has a short replicative cycle (1–2 hours in tumor cells) and is not endemic to the North American population; all of these properties enhance its therapeutic potential. An additional, previously unknown feature of VSV is its 'leukemolytic' property, shown here. The ability of VSV to purge bone marrow cultures *ex vivo* may have substantial consequences clinically in autologous bone marrow transplantation.

Methods

Cell lines and virus. The Indiana serotype of VSV was used throughout this study and was propagated in 1929 cells. The following cell lines and corresponding culture media were used: human foreskin fibroblasts (AG 1522; α MEM with 10% FBS); primary normal human fibroblasts established at the Ottawa Regional Cancer Centre from forearm biopsies (OSF7, 12 and 16; α MEM with 10% FCS); primary human prostate epithelial cells (PrEC 5; Clonetics, San Diego, California; manufacturer's instructions were followed); primary human ovarian surface epithelial cells (H05E; provided by L. Macdonald and B. Vanderhyegh; DMEM with 15% FBS, 5 μ g/ml insulin, 10 μ g/ml transferrin and 5 ng/ml epidermal growth factor); human ovarian carcinomas (A2780; DMEM/F12 with 10% FBS); human ovarian carcinomas (OVCA 420 and 432; α MEM with 10% FBS, 2 mM α -glutamine, 1% sodium pyruvate and 1% non-essential amino acids); human ovarian carcinoma (C13; RPMI 1640 with 10% FBS); human melanoma (SK-MEL3; DMEM/F12 HAM with 10% FBS); human lung carcinoma (LC80; α MEM with 10% FBS); human colon carcinoma (HCT116; DMEM with 10% FBS); and human prostate carcinoma (LNCaP; α MEM with 10% FBS).

Viral production and cytopathic effect in tumor cells and primary fibroblasts. Cell lines and primary fibroblasts were seeded to 80% confluence in 35-mm dishes containing 2 ml α MEM (Life Technologies) supplemented with 10% FBS, and either were not treated or were supplemented with various doses of interferon, then incubated for 16 h. Virus diluted in medium to an appropriate MOI was added to the dishes and allowed to adsorb for 30 min at 37 °C. The dishes were subsequently rinsed three times with PBS overlaid with 2 ml medium and incubated for 8 h. Titers in virus-laden medium from these dishes were then determined as described²³ with minor modifications. Virus-containing medium was serially diluted in α MEM medium supplemented with 10% FBS. Each virus inoculum was allowed to adsorb to a monolayer of L cells for 30 min at 37 °C and was then overlaid with 0.5% agarose in medium. After an overnight incubation at 37 °C, the agar was removed and the monolayers were fixed in 4% paraformaldehyde and stained with 0.5% methylene blue. Plaques were then counted.

The cytopathic effect was monitored in infected, live monolayers grown on coverslips and infected as described above. At a series of 12-hour time points after infection, phase contrast micrographs of monolayers were obtained using a Zeiss Axiovert light microscope.

Kinetics of cytolysis VSV. Various cell lines were grown in 12-well plates to approximately 75% confluency. Medium was replaced with fresh medium with or without interferon α (100 units/ml INTRON-A; Shering, Kenilworth, New Jersey) and incubated for another 12 h before infection. The cells were infected for 30 min at 37 °C with VSV in 20 μ l medium at an MOI of 0.1 PFU per cell, after which fresh medium was added to fill each well. At 12-hour time points, medium was removed and cells were fixed with methanol and stained with methylene blue and eosin.

Mixed cell cultures. Equal numbers of normal human fibroblasts and 293T cells were co-cultured on gelatin-coated coverslips in α -MEM (10% FBS with or without 100 U/ml interferon- α) and were infected with VSV at an MOI of 0.1 PFU/cell. At 18 h after infection, the supernatants were collected and the cells were fixed with methanol. A polyclonal antibody against large T antigen was hybridized to the fixed cells and detected with CY3-conjugated goat secondary antibody against rabbit (Sigma). Nuclei were stained with DAPI (4,6-diamidino-2-phenylindole). The coverslips were mounted in 'anti-fade' and viewed under a fluorescence microscope.

Hematopoietic cell culture. White blood cells were isolated from normal human marrow and exposed to virus at various multiplicities of infection for 30 min. The cells were then washed three times with PBS and incubated overnight in IMDM with 20% FBS and 10% medium conditioned by bladder carcinoma cell line 5637. Medium was then collected and titers were determined as described above.

The leukemic cell line OCI/AML3 was cultured in IMDM with 10% FBS, whereas OCI/AML4 and OCI/AML5 cells were cultured in IMDM with 10% FBS and 10% 5637-conditioned medium (OCI/AML3, 4 and 5 cells provided by M. Minden). The dose of virus that caused 50% killing at both 24 and 48 h was determined using the MTS assay, which measures the bioreduction of a tetrazolium compound (MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt) into a formazan, which can be measured colorimetrically.

The growth-factor-independent cell line OCI/AML3 was mixed at a ratio of 1:9 with normal human marrow and was co-cultured in IMDM with 20% FBS and 10% 5637-conditioned medium. Test cultures were infected at an MOI of 1.0 PFU/cell or 5 PFU/cell, whereas controls were left uninfected. At 24 h after infection, cells were plated in methylcellulose containing 12% FBS with or without growth factors (10% 5637-conditioned medium, kit (stem cell factor) ligand and erythropoietin). Colonies were counted 14 d later.

VSV treatment of xenograft tumors. Xenografts were initiated in female BALB/c nude mice 8–10 weeks old by injecting 5×10^5 SK-MEL 3 human melanoma cells subcutaneously in each of two sites per mouse. When tumors reached 4–5 mm in diameter, they were either left untreated or treated with a single intratumoral injection of either 1×10^6 PFU VSV in culture medium or 2.5×10^5 VSV-infected SK-MEL 3 cells (infected at an MOI of more than 10 PFU/ml for 2 h). Alternatively, CD-1 nude mice were 'seeded' on one flank with SK-MEL 3 cells as described above. Tumors 4–5 mm in diameter were injected with 1×10^6 PFU live VSV or VSV inactivated by UV irradiation. Murine interferon alpha was administered by intraperitoneal injection (PBL Biomedical Laboratories, New Brunswick, New Jersey). The health of the mice was monitored and tumor sizes were measured daily.

Statistical analysis. For single comparisons (Fig. 3a, day 6), the tumor volumes were analyzed by Student's *t*-test. For multiple comparisons (Fig. 3a, days 3, 4 or 5), the data were first analyzed by one-way analysis of variance, and statistical significance was determined by Bonferroni's multiple comparison test using Prism 3.0 software (Graphpad Software, San Diego, California).

Acknowledgments

We thank D. Gray for aid in preparation and evaluation of the manuscript. This work was funded by a grant from the National Cancer Institute of Canada with funds from the Canadian Cancer Society and by the Ottawa Regional Cancer Centre Foundation. D.F.S. was supported by an Ontario Graduate Scholarship in Science and Technology (OGSST).

RECEIVED 6 DECEMBER 1999 ACCEPTED 25 APRIL 2000

1. Stark, G.R., Kerr, I.M., Williams, B.R., Siverman, R.H. & Schreiber, R.D. How cells respond to interferons. *Annu. Rev. Biochem.* **67**, 227-264 (1998).
2. Koke, O. & Niederle, N. Development and mechanisms of interferon resistance. *Cancer Treat. Rev.* **17 Suppl A**, 81-88 (1999).
3. Colamonici, O.R., Domanski, P., Ratanias, L.C. & Diaz, M.O. Correlation between interferon (IFN) alpha resistance and deletion of the IFN α/β genes in acute leukemia cell lines suggests selection against the IFN system. *Blood* **80**, 744-749 (1993).
4. Xu, B., Grandér, D., Sangfelt, O. & Einhorn, S. Primary leukemia cells resistant to alpha-interferon in vitro are defective in the activation of the DNA-binding factor interferon-stimulated gene factor 3. *Blood* **84**, 1942-1949 (1994).
5. Wang, L.H. et al. Interferon-resistant human melanoma cells are deficient in ISGF3 components: STAT1, STAT2, and p48/ISGF3gamma. *J. Biol. Chem.* **272**, 25779-25785 (1997).
6. Avril, E. et al. Characterization of a gastric tumor cell line defective in MHC class I inducibility by both alpha- and gamma-interferon. *Tissue Antigens* **47**, 391-396 (1996).
7. Sun, W.H. et al. Interferon-alpha resistance in a cutaneous T-cell lymphoma cell line is associated with lack of STAT1 expression. *Blood* **91**, 570-576 (1998).
8. Latchworth, G.J., Rodríguez, L.L. & Del Cid, R. Vesicular stomatitis virus. *J. Virol.* **157**, 239-280 (1999).
9. Belkowsky, L.S. & Sen, G.C. Inhibition of vesicular stomatitis viral mRNA synthesis by interferons. *J. Virol.* **61**, 653-660 (1987).
10. Burke, F. Cytokines (IFN γ , TNF α , IL-2 and IL-12) and animal models of cancer. *Cytokines Cell. Mol. Ther.* **5**, 51-61 (1998).
11. Thomsen, A.R. et al. Cooperation of B cells and T cells is required for survival of mice infected with vesicular stomatitis virus. *Int. Immunol.* **9**, 1757-1766 (1997).
12. Huneycutt, B.S., B. Z., Aoki, C.J. & Reiss, C.S. Central neuropathogenesis of vesicular stomatitis virus infection of immunodeficient mice. *J. Virol.* **67**, 6668-6705 (1993).
13. Coukos, G. et al. Use of carrier cells to deliver a replication-selective herpes simplex virus-1 mutant for the intraperitoneal therapy of epithelial ovarian cancer. *Clin. Cancer Res.* **5**, 1523-1537 (1999).
14. Smith, R.R., Huebner, R.J., Rowe, W.P., Schatten, W.E. & Thomas, L.B. Studies on the use of viruses in the treatment of carcinomas of the cervix. *Cancer* **9**, 1211-1218 (1956).
15. Lorence, R.M. et al. Complete regression of human fibrosarcoma xenografts after local Newcastle disease virus therapy. *Cancer Res.* **54**, 6017-6021 (1994).
16. Lorence, R.M. et al. Complete regression of human neuroblastoma xenografts in athymic mice after local Newcastle disease virus therapy. *J. Natl. Cancer Inst.* **86**, 1229-1233 (1994).
17. Rothmann, T., Hengstlermann, A., Witaker, N.J., Scheffner, M. & zur Hausen, H. Replication of ONYX-015, a potential anticancer adenovirus, is independent of p53 status in tumor cells. *J. Virol.* **72**, 9470-9478 (1998).
18. Shimada, A. et al. Aberrant expression of double-stranded RNA-dependent protein kinase in hepatocytes of chronic hepatitis and differentiated hepatocellular carcinoma. *Cancer Res.* **53**, 4434-4438 (1993).
19. Strong, J.E., Coffey, M.C., Tang, D., Sabarin, P. & Lee, P.W. The molecular basis of viral oncology: usurpation of the Ras signaling pathway by reovirus. *EMBO J.* **17**, 3351-3362 (1998).
20. Cassidy, K.A., Gross, M. & Rizmán, B. The herpes simplex virus US11 protein effectively compensates for the gamma1(34.5) gene if present before activation of protein kinase R by precluding its phosphorylation and that of the alpha subunit of eukaryotic translation initiation factor 2. *J. Virol.* **72**, 8520-8526 (1998).
21. Cave, D.R., Hendrickson, E.M. & Huang, A.S. Defective interfering virus particles modulate virulence. *J. Virol.* **55**, 366-373 (1981).

VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents

David F. Stojdl,¹ Brian D. Lichty,¹ Benjamin R. tenOever,² Jennifer M. Paterson,^{1,6} Anthony T. Power,^{1,6} Shane Knowles,¹ Ricardo Marius,¹ Jennifer Reynard,¹ Laurent Poliquin,³ Harold Atkins,¹ Earl G. Brown,⁶ Russell K. Durbin,⁴ Joan E. Durbin,⁴ John Hiscott,^{2,5} and John C. Bell^{1,6,*}

¹Ottawa Regional Cancer Centre Research Laboratories, 501 Smyth Road, Ottawa, Ontario, Canada K1H 8L6.

²Terry Fox Molecular Oncology Group, Lady Davis Institute for Medical Research, McGill University, Montreal, Quebec, Canada

³Department of Biological Sciences, Université du Québec à Montréal, P.O. Box 8888, Station Centre-ville, Quebec, H3C 3P8, Montreal, Canada

⁴Children's Research Institute, Children's Hospital, Columbus, Department of Pediatrics, College of Medicine and Public Health, The Ohio State University, Columbus, Ohio 43205

⁵Departments of Microbiology and Immunobiology, Medicine, McGill University, Montreal, Quebec, Canada H3T 1E2

⁶Department of Biochemistry, Microbiology and Immunology, University of Ottawa, 451 Smyth Road, Ottawa, Ontario, Canada K1H 8M5

*Correspondence: John.bell@orcc.on.ca

Summary

Ideally, an oncolytic virus will replicate preferentially in malignant cells, have the ability to treat disseminated metastases, and ultimately be cleared by the patient. Here we present evidence that the attenuated vesicular stomatitis strains, AV1 and AV2, embody all of these traits. We uncover the mechanism by which these mutants are selectively attenuated in interferon-responsive cells while remaining highly lytic in 80% of human tumor cell lines tested. AV1 and AV2 were tested in a xenograft model of human ovarian cancer and in an immune competent mouse model of metastatic colon cancer. While highly attenuated for growth in normal mice, both AV1 and AV2 effected complete and durable cures in the majority of treated animals when delivered systemically.

Introduction

Over the last decade, a variety of replicating oncolytic viruses have been selected or engineered to be therapeutics that exploit genetic defects unique to tumor cells (reviewed in Bell et al., 2002; Gromeier and Wimmer, 2001; Hawkins et al., 2002; Krutz and Curiel, 2002; Norman et al., 2000). One genetic defect frequently arising during tumor evolution, is diminished interferon (IFN) responsiveness (Bello et al., 1994; Linde et al., 1995; Lu et al., 2000; Martin et al., 2001; Sun et al., 1998; Wong et al., 1997). This reflects the important role that interferon-regulated pathways play in the control of normal cell growth and apoptosis. Interferon is also a key mediator of the individual cell's antiviral response and thus tumor cells, which acquire mutations allowing them to escape interferon-mediated growth control programs, will simultaneously compromise their innate antiviral response. We hypothesized that viruses whose replication is inhibited by interferon should grow well in tumor but not normal cells. We and others have found that vesicular stomatitis virus (VSV), whose growth is strongly inhibited by interferon, is a

potent oncolytic virus (Balachandran and Barber, 2000; Stojdl et al., 2000b). In fact, while VSV infections are uniformly fatal to nude mice (Huneycutt et al., 1993; Stojdl et al., 2000b), we found that prophylactic interferon treatment can rescue even immunocompromised animals while preserving virus-mediated oncolysis. We reasoned that a virus that both induces the production of interferon and is susceptible to its antiviral effects would be a superior therapeutic. Here we describe two naturally occurring VSV variants that possess both these properties. The VSV variants retain oncolytic activity in vitro and in a variety of in vivo models but because of their potent induction of interferon have a vastly improved therapeutic index over their wild-type (WT) counterpart.

Results

Attenuation of VSV in vivo is dependent upon intact interferon signaling pathways

Two variants of VSV that produce small plaques on interferon-responsive cells (herein referred to as AV1 and AV2) were found

SIGNIFICANCE

A key limitation to the application of viruses as cancer therapeutics is the possibility of uncontrolled virus growth in normal tissues, potentially leading to treatment complications or disease. Here, we describe novel, oncolytic variants of vesicular stomatitis virus (VSV) that not only have potent anti-tumor activity in vivo, but establish an anti-viral state that protects against the toxicity associated with infection of healthy cells. Our work has uncovered the mechanism that virulent VSV strains use to defeat host antiviral defences, furthering our understanding of early IFN signaling in response to a viral invader. These findings have directed us toward the development of improved VSV-based oncolytic viruses and are generally applicable to a wide range of viral based therapeutics.

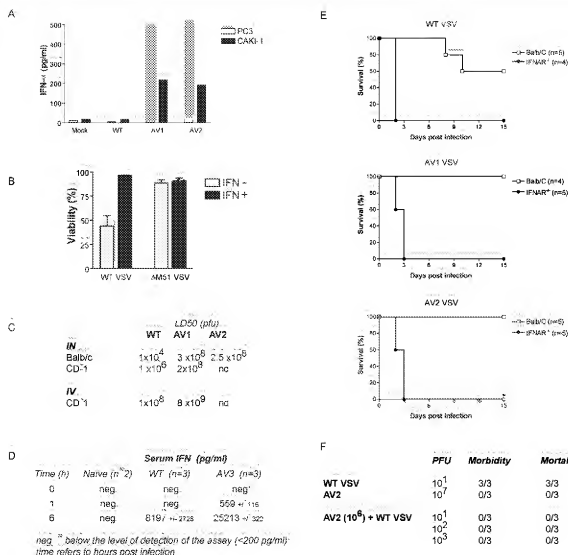


Figure 1. Decreased in vivo toxicity of AV1 and AV2 is mediated by interferon

A: Human prostate carcinoma cells (PC3) and human renal carcinomas cells (CAK1-1) were either mock infected or infected with wild-type (WT), AV1, or AV2 strains of VSV. Culture media were assayed by EUSA to detect human IFN- α production 48 hr post-infection.

B: Exogenous interferon is required to protect MEFs from WT GFP but not from AV3 GFP. Balb/C MEFs were pretreated with 0 or 30 U/ml universal type I interferon for 16 hr and then infected with WT GFP VSV or AV3 GFP at an MOI of 0.1. Twenty-four hours later, cell viability was measured by MTS assay.

C: In vivo toxicity of WT versus mutant VSV strains by route and mouse strain. IN = intranasal; IV = intravenous; nd = not determined.

D: AV3 induces IFN- α quicker and to a greater degree than WT VSV in vivo. Groups of mice were either mock infected or infected with WT GFP or AV3 GFP, and their serum IFN- α levels were assayed at the indicated times post-infection.

E: Balb/C and Balb/C *INFAR*^{-/-} mice were infected intranasally with WT VSV, AV1, or AV2 virus and monitored for morbidity.

F: AV2 can protect mice against lethal WT VSV infection. *PKR*^{-/-} mice were infected intranasally at various doses with either WT, AV2, or both and monitored for morbidity or mortality. Values denote number of mice per group.

to induce from twenty to fifty times more interferon α (IFN- α) than WT VSV following infection of epithelial cell lines (Figure 1A). Sequencing of the variants revealed that they differed from the wild-type strain in their M proteins with a single amino acid substitution in the case of AV1 (M51R) and two amino acids (V221F and S226R) in AV2. A third variant was created to be a mimetic of AV1 by complete deletion of methionine 51 (M Δ 51 or AV3) and found to have biological properties indistinguishable from AV1 and AV2. As expected (Stojdl et al., 2000b), primary mouse embryo fibroblasts are protected against WT VSV infec-

tion only in the presence of exogenously added interferon whereas MEFs (mouse embryonic fibroblasts) are refractory to infection by the interferon inducing mutant AV3 (Figure 1B).

In animals, the role of the interferons in protecting against virus infection and the mechanisms underlying their induction are more complex than in the simple tissue culture systems described above (Barchet et al., 2002; Levy, 2002). Nevertheless, we show in the following that the AV strains are more potent interferon inducers and have reduced toxicity in mice in a strictly interferon-dependent fashion. For example, mice

infected intravenously can tolerate some 80 times more AV1 virus than WT VSV, and AV3 induced a more rapid and robust production of interferon than WT VSV (Figures 1C and 1D). The critical role that interferon signaling plays in the protection of mice from infection by AV1 and AV2 was verified using interferon receptor knockout (*IFNAR*^{-/-}) animals. The LD₅₀ of AV1 and AV2 when delivered intranasally to Balb/C (*IFNAR*^{+/+}) mice was determined to be 10,000 times greater than WT VSV delivered by the same route (Figure 1C). Similar results were seen in CD-1 mice (WT = 1×10^5 ; AV1 = 2×10^6 pfu). However, in the absence of a functional interferon receptor, AV1 and AV2 were as toxic as wild-type virus, indicating that the attenuation of AV1 and AV2 growth in vivo is dependent upon an intact interferon system (Figure 1E).

The AV1 and AV2 variants protect mice from infection by WT VSV

Mice that lack the double-stranded RNA-dependent kinase (PKR) gene are known to be exquisitely sensitive to infection by wild-type vesicular stomatitis virus, although PKR^{-/-} fibroblasts can be protected by prophylactic treatment with interferon (Balachandran et al., 2000). Since AV1 and AV2 strongly induce interferon production during the course of a natural infection, we tested whether PKR^{-/-} mice would be resistant to infection by these viruses. Indeed we found that while <10 pfu of wild-type VSV can kill PKR^{-/-} mice (Figure 1F), doses greater than 10^7 pfu of AV1 and AV2 were well tolerated by PKR^{-/-} animals. More strikingly, when coinfecting with AV2, the LD₅₀ of wild-type VSV was dramatically increased in PKR^{-/-} animals. Indeed, doses 100 times greater than the LD₅₀ for WT VSV were well tolerated when coinfecting with AV2 (Figure 1F). Given that PKR^{-/-} fibroblasts can be protected from WT VSV infection by prophylactic interferon administration (Balachandran et al., 2000), that the AV variants induce interferon, and that AV variants are toxic in mice that lack a functional interferon receptor, we believe that the protective effect of AV2 on PKR^{-/-} mice is most easily explained by the ability of these viruses to strongly induce interferon production in the infected animal.

Wild-type, AV1 and AV2 viruses trigger antiviral responses in infected cells

We used microarray and Western blot analysis over a time-course of virus infection to allow us to detect early signaling events triggered by WT and AV variants that lead to the transcriptional activation of antiviral genes. Others have established that an early response to virus infection is the phosphorylation and activation of the latent transcription factor IRF-3 (Sato et al., 1998b). It appears that WT, AV1 and AV2 viruses trigger IRF-3 phosphorylation with similar kinetics (Figure 2A). Following phosphorylation, IRF-3 assembles together with CBP/300 and, along with other transcription factors (e.g., NFκB and c-JUN/ATF-2), initiates the transcription of a number of antiviral gene products (Wathelet et al., 1998). As shown in Table 1, microarray analysis revealed that a large number of genes were dramatically induced 3 hr post-infection with all three viruses. Many of these genes are known to be activated by virus infection (Nakaya et al., 2001), including several that are directly regulated following activation of the latent transcription factors IRF-3, NFκB, and c-JUN/ATF-2 (Genin et al., 2000). We validated the microarray data by performing RT-PCR analysis on a sampling of gene products (Figure 2B).

In Figures 2C–2E, we present a model in which virus infection leads to waves of transcriptional events that are sequential and interdependent. For example, genes that we refer to herein as primary response genes were induced 3–6 hr post-infection by all three viruses (Figures 2B and 2C). On the other hand, secondary response genes that require the production of IFN-β protein and the autocrine activation of the JAK/STAT pathway (Figure 2D) were differentially induced by the wild-type and attenuated viruses (see IRF-7 in Figures 2A and 2D). As a consequence of the impaired IRF-7 production in WT VSV-infected cells, tertiary response gene products like the IFN-α transcripts were not induced in wild-type VSV-infected cells (Figure 2E). These results indicate that all three viruses trigger activation of IRF-3 and the subsequent transcription of a cohort of genes that we call primary response genes. We hypothesized that the M protein encoded by wild-type VSV disables the host cell's antiviral response by disrupting subsequent activation of secondary and tertiary response genes.

VSV M protein blocks the nuclear export of interferon-β mRNA

It has been suggested that VSV M protein either blocks the transcription of the *IFN-β* gene (Ahmed et al., 2003; Ferran and Lucas-Lenard, 1997), inhibits the nuclear export of mRNAs (Her et al., 1997; von Kobbe et al., 2000), or interferes with JAK/STAT signaling (Terstegen et al., 2001). Our transcript profiling studies would be consistent with either of the latter two mechanisms; however, we have been unable to show any impairment in the induction of the JAK/STAT pathway by exogenous interferon in infected cells (data not shown). On the other hand, when we used microarray or RT-PCR analysis to compare and contrast transcripts in nuclear and cytoplasmic fractions, we found clear differences between wild-type and attenuated virus-infected cells (Figure 3A). Importantly, *IFN-β* mRNA although induced in nuclear fractions by all three viruses was not found in the cytoplasmic pool of mRNAs in WT infected cells. Furthermore, *IFN-β* was undetectable in culture media from cells infected with WT VSV, while copious amounts of the cytokine were produced from cells infected with either AV1 or AV2 (Figure 3B). Two additional experiments help shed light upon how WT VSV subverts the interferon signaling pathway. First, cells were infected with either WT or AV3 VSV, and at 22 hr post-infection, *IFN-α* production in tissue culture supernatant was measured. WT VSV does not induce the production of *IFN-α* while AV3 is a potent inducer (Figure 3C). The induction of *IFN-α* by AV3 was dependent upon prior production of *IFN-β* as inclusion of neutralizing anti-*IFN-β* antibody to infected cultures inhibited *IFN-α* production from these cells (Figure 3C). Second, we constructed a wild-type VSV that expresses a constitutively active version of IRF-7. This virus has an attenuated phenotype and induces the expression of *IFN-α* genes within 4 hr post-infection, even in the presence of wild-type VSV M protein (Figure 2D). In total, these results are consistent with the idea that wild-type VSV triggers a primary antiviral response, but through coordinate expression of viral gene products blunts secondary and tertiary responses by blocking nuclear export of critical antiviral mRNAs.

AV1 and AV2 retain their ability to kill tumor cells in vitro and in vivo

To assess the oncolytic properties of the attenuated VSV strains, the NCI human tumor cell panel (60 cell lines from a spectrum

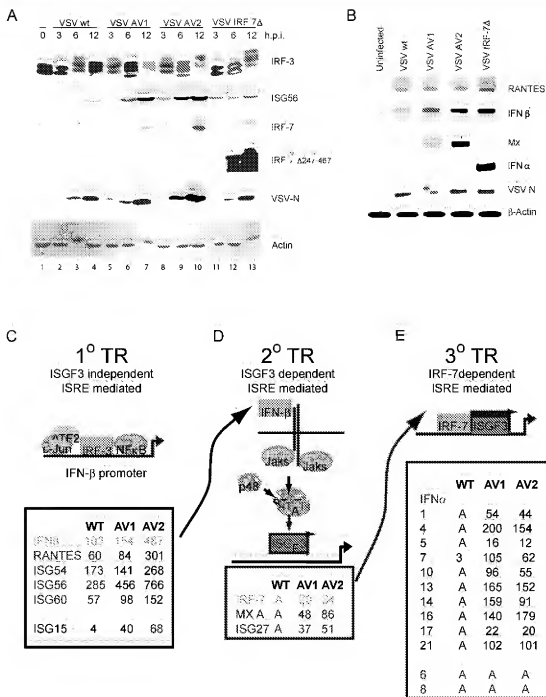


Figure 2. The secondary transcriptional response is inhibited by WT VSV but not AV1 or AV2

A: Western blot analysis showed similar kinetics of IRF-3 activation between WT and mutant VSVs; however, ISG56 (primary response) protein expression was severely impaired in WT infected cells. IRF-7 protein is detected only in AV1- and AV2-infected cells. IRF-7Δ appears to be able to induce the expression of endogenous IRF-7.

B: RT-PCR data at 4 hr post-infection of A549 cells showed primary response genes *RANTES* and *IFN-β* induced to similar levels in WT and mutant VSV-infected cells while upregulation of *MX1* (secondary response) was impaired in WT infected cells.

C: Primary response to viral infection is mediated by IRF-3, cJUN/ATF-2, and NFκB (shown here forming part of the enhancosome complex at the *IFN-β* promoter). Microarray data indicate primary transcriptional response genes robustly upregulated in both WT and mutant virus-infected cells. (a: *ISG15* is known to require ISGF3 for full induction). Values represent fold induction over mock infected.

D: IFN-β is then translated and secreted to stimulate, in an autocrine fashion, JAK/STAT signaling to form ISGF3 complexes in the nucleus, which mediates the induction of genes of the secondary transcriptional response. While cells infected with AV1 or AV2 show robust upregulation of these genes, WT infected cells show no expression at all [A = absent].

E: Without the consequent expression of IRF-7 in cells infected with WT VSV, the tertiary transcriptional wave, which includes almost all *IFN-α* genes, cannot take place [b: *IFN-α7* is marginally detected by the array in WT samples]. In contrast, AV1- and AV2-infected cells efficiently induce the expression of *IFN-α* genes.

Table 1. Microarray analysis of the transcriptional response to VSV infection over time

Accession #	Common name	WT			AV1			AV2		
		Hours post infection			Hours post infection			Hours post infection		
		3	6	12	3	6	12	3	6	12
Primary transcriptional response										
NM_000201.1	CD54	2.3	A	A	3.3	3.0	33.6	2.7	5.4	56.8
NM_016323.1	CEB1	2.0	21.2	204.4	1.6	38.0	279.3	1.9	73.6	490.5
U83981	GADD34	10.8	57.5	95.0	3.1	48.8	422.7	5.5	159.0	686.7
NM_002176.1	IFN beta	4.2	103.2	408.6	3.2	154.5	721.9	3.6	487.3	2157.9
NM_000620.1	IL6	7.3	19.1	38.2	3.8	44.7	171.6	7.4	120.3	238.7
BE88744	ISG54	19.5	173.1	804.5	4.7	141.0	721.9	11.4	268.4	1357.8
NM_001548.1	ISG56	32.3	285.9	855.2	20.0	456.8	1411.7	39.8	766.0	1992.1
NM_001549.1	ISG60	7.6	57.5	238.0	4.1	97.7	288.6	7.0	151.6	457.7
AF063612.1	OASL	6.6	71.8	222.0	3.4	81.9	388.9	5.7	172.0	776.9
NM_021127.1	PMAIP1	5.2	22.1	58.6	2.1	17.3	87.5	4.3	34.3	169.8
NM_002852.1	PTX3	5.9	3.1	A	6.4	11.7	114.0	4.9	29.9	117.6
AF332558.1	PUMA	10.6	A	A	A	38.7	211.6	9.8	77.8	428.0
NM_002985.1	RANTES	3.4	60.1	945.0	2.6	84.9	1796.7	4.1	301.8	3916.1
AY029180.1	SUPAR	3.7	9.8	14.7	2.4	10.5	40.5	2.8	27.7	46.3
NM_006290.1	TNFAIP3	2.8	6.3	15.5	2.7	13.8	83.5	3.1	30.5	152.8
Secondary transcriptional response										
NM_030641.1	APOL6	A	A	A	A	15.3	40.8	A	25.2	37.3
AF323540.1	APL1L	1.8	A	A	1.0	11.3	25.7	2.2	10.7	34.5
U94487	CX3C chemokine precursor	2.0	2.5	2.5	1.7	7.0	45.1	2.3	14.1	65.9
BC002666.1	GBP1	A	A	4.2	A	35.9	171.6	1.4	66.2	249.2
NM_006018.1	HM74	A	A	A	2.2	29.1	72.5	A	66.4	45.4
NM_031212.1	HMR53/4	A	A	A	2.5	4.2	21.3	A	10.1	18.3
NM_005531.1	IFI16	A	A	A	2.4	12.8	38.1	2.8	18.9	46.2
NM_005532.1	IFI27	A	A	21.9	A	36.6	281.0	A	51.0	295.4
NM_004509.1	IFI41	A	A	A	A	10.0	22.8	1.3	11.9	18.1
NM_022873.1	IFI6-16	0.9	2.5	2.2	0.7	7.0	15.6	1.1	9.6	15.7
NM_003641.1	IFITM1	1.9	A	A	1.2	8.5	67.1	1.9	14.3	42.1
AA749101	IFITM1	1.2	3.1	2.3	1.0	6.6	40.9	1.2	9.5	32.6
NM_000882.1	IL12A	1.6	A	A	A	4.9	13.7	A	6.5	28.8
M15329.1	IL1A	nd	A	A	A	8.3	79.4	A	27.0	287.6
NM_004030.1	IRF7	1.4	A	A	A	19.9	109.9	2.2	33.7	144.3
NM_006084.1	IRF9	A	A	1.2	1.6	6.5	11.2	1.5	7.8	17.4
BC001356.1	ISG35	1.1	A	A	1.0	5.8	23.2	1.4	7.1	20.2
AF280094.1	ISG75	1.3	1.8	2.2	1.5	10.3	16.2	1.2	12.5	13.8
AF280094.1	ISG75	0.9	1.7	A	1.2	7.5	10.8	1.5	9.5	11.1
U17496.1	LMP7	A	A	A	A	7.6	15.3	0.9	10.0	10.4
NM_006417.1	MTAP44	A	A	23.3	A	10.8	82.7	A	18.0	133.9
NM_002462.1	MX A	A	A	27.6	A	48.1	261.9	A	85.7	232.9
AB014515	NEDD4 BP1	A	A	9.2	2.0	4.0	13.1	1.5	4.5	19.5
NM_002759.1	PKR	0.5	0.9	2.0	0.8	4.3	15.2	1.0	6.6	9.6
NM_021105.1	PLSCR1	1.4	1.7	A	2.2	5.0	24.9	2.1	4.9	15.1
NM_017912.1	putative Ub ligase	A	A	19.1	A	9.6	26.3	A	12.5	24.8
BF939675	SECTM1	A	A	A	A	20.7	93.8	A	24.8	33.9
BC004395.1	Similar to apolipoprotein L	A	A	A	A	11.7	17.2	A	14.6	21.1
NM_003141.1	SSA1	A	A	A	1.2	5.9	11.2	1.4	7.9	11.3
AA083478	STAF50	nd	A	nd	1.7	8.5	96.3	nd	16.7	56.1
NM_005419.1	STAF2	1.1	A	A	1.3	3.0	9.1	1.1	4.3	9.1
NM_003810.1	TRAIL	A	A	A	A	22.4	135.4	0.7	37.3	88.6
NM_020119.1	ZAP	A	A	19.4	0.9	4.6	79.9	A	11.4	133.8
Tertiary transcriptional response										
M12350.1	IFN-27	A	A	A	nd	nd	102.3	nd	A	101.4
NM_024013.1	IFNA1	nd	A	A	nd	2.2	53.6	nd	A	44.0
NM_002171.1	IFNA10	A	A	A	A	A	96.2	A	A	55.1
NM_006900.2	IFNA13	A	A	A	A	A	165.4	nd	A	152.1
NM_002172.1	IFNA14	A	A	A	A	A	159.0	A	4.4	91.1
NM_002173.1	IFNA16	1.0	A	1.8	1.1	0.9	139.9	0.7	3.3	95.7
M38289.1	IFNA17	A	A	A	A	A	21.5	A	A	19.8
NM_002169.1	IFNA5	1.0	A	A	0.9	A	16.4	0.9	A	11.6
NM_021057.1	IFNA7	A	A	3.5	A	A	105.0	A	3.4	61.7

Data represented as fold change compared to mock-infected samples. All samples are from nuclear fractions of infected cells.

A = Absent (no detectable mRNA); nd = no data; bold genes represent "archetypal genes," see text for explanation.

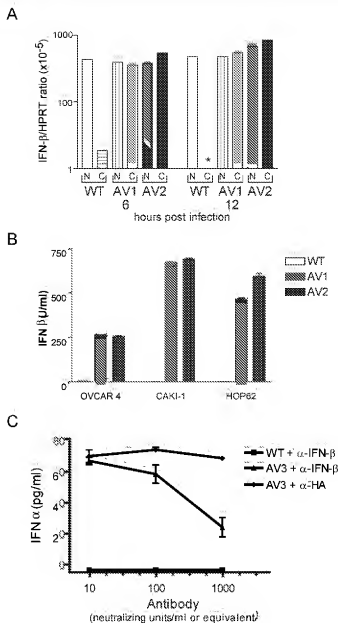


Figure 3. WT VSV inhibits IFN- β mRNA nuclear/cytoplasmic transport and blocks IFN- α production

A: IFN- β mRNAs are severely depleted in cytoplasmic fractions from WT VSV-infected cells as determined by quantitative RT-PCR. Nuclear (N) and cytoplasmic (C) total RNA fractions from cells infected with WT, AV1, or AV2 VSV were assayed for IFN- β mRNA, normalized to HPRT mRNA from the same sample. * indicates no IFN- β mRNA detected.

B: Cells infected with either WT or mutant VSV strains were assayed by ELISA for IFN- β production. AV1- and AV2-infected cells and not WT VSV-infected cells produce secreted IFN- β .

C: Blocking IFN- β inhibits the production of IFN- α . OVCAR-4 cells were infected with mutant VSV in the presence of neutralizing antibody to IFN- β or an irrelevant antibody and subsequently assayed by ELISA for IFN- α production.

of malignancies) was challenged with either WT, AV1, or AV2 viruses and assayed for metabolic cell death 48 hr later. It is clear from Table 2A that WT VSV is able to infect and kill a wide range of cancer cell types, and furthermore, the majority of cancer cell lines tested demonstrated impaired responses to either IFN- α or IFN- β (Table 2B). AV1 and AV2 were as effective at killing these interferon nonresponsive tumor cell lines as WT VSV.

We previously reported the successful treatment of subcutaneous xenograft tumors in nude mice with WT VSV; however, in these experiments, exogenous interferon was required to protect the animals from succumbing to viral infection. Our results with the NCI cell panel suggest that AV1 and AV2 should efficiently kill tumor cells with little toxicity in mouse models even in the absence of interferon treatment, and therefore we conducted an extensive analysis of the *in vivo* oncolytic properties of the AV variants. In a first series of experiments, human ovarian carcinoma cells were injected into the peritoneal cavity of CD-1 nude mice and allowed to grow for 12 days. Mice (14/15) receiving UV-inactivated virus developed ascites by day 15 post-treatment. In contrast, three doses of AV2 delivered into the peritoneal cavity provided durable cures in 70% of the mice (Figure 4A). Remarkably, while a single therapeutic dose of WT VSV is uniformly lethal to nude mice (Stojdl et al., 2000b), none of the animals treated with three doses of AV2 exhibited even symptoms of virus infection.

Systemic treatment in immune-competent mouse models

Earlier preclinical, clinical, and mathematical modeling studies (Wein et al., 2003) predict that greatest anti-tumor efficacy is achieved when the delivered virus is distributed diffusely throughout the tumor (e.g., through tumor vasculature). Given that certain oncolytic viruses are rapidly inactivated in blood or inhibited by physical barriers (Ikeda et al., 2000; Wakimoto et al., 2002, 2003; Yoon et al., 2001), we felt it was important to determine the minimum VSV doses required to achieve effective delivery of VSV into tumor sites. For these studies, we engineered a VSV strain to express GFP during productive infections and examined subcutaneous tumors 24 hr after intravenous virus administration. Virus doses in the range of 10^5 – 10^9 pfu per mouse gave optimum tumor delivery (Figure 5). In other experiments and those shown below, we found that virus administered in this dose range also provided maximum therapeutic benefit to tumor-bearing animals. For example, subcutaneous tumors were established by injecting CT26 colon carcinoma cells into the hind flank of syngeneic Balb/c mice. Once tumors became palpable (approximately 10 mm³), virus was administered via tail vein injection. Twelve days post-treatment, mice receiving UV-inactivated VSV reached endpoint with an average tumor size of 750 mm³. In contrast, a single treatment with AV2 showed significant efficacy, delaying the time to endpoint by almost 3-fold (34 days). Of the eight animals in this treatment group, seven were considered partial responders while only one mouse did not respond to the treatment (data not shown). When multiple doses of AV1 or AV2 were given intravenously, the efficacy of the treatments was markedly increased (Figure 4B). With the exception of one animal, all tumors responded to treatment with AV1, with 3/6 mice showing complete tumor regression. Two of these mice showed complete regressions as early as day 8 and 9, respectively, post-infection. Two of the re-

Table 2A. Mutant VSV strains are highly lytic on members of the NCI 60 panel of cancer cell lines

	WT	AV1		AV2	
		MOI	MOI	MOI	MOI
Leukemia	67% (4/6)*	0.13	nd	60% (3/5)	0.02
NSC lung carcinoma	78% (7/9)	0.02	60% (3/5)	75% (6/8)	0.19
Colon carcinoma	86% (6/7)	0.037	100% (5/5)	100% (6/6)	0.017
CNS	80% (4/5)	0.02	50% (1/2)	60% (3/5)	0.38
Melanoma	75% (6/8)	0.1	100% (2/2)	63% (5/8)	0.25
Ovarian carcinoma	100% (6/6)	0.3	67% (2/3)	60% (3/5)	0.14
Renal carcinoma	88% (7/8)	0.24	100% (3/3)	100% (7/7)	0.48
Prostate	100% (2/2)	0.06	100% (2/2)	100% (2/2)	0.04
Breast	83% (5/6)	0.009	75% (3/4)	60% (3/5)	0.12
All cell lines tested	82% (47/57)	0.11	80% (21/26)	75% (38/51)	0.20

*Percent of NCI 60 panel cell lines by tumor type deemed highly sensitive to virus infection. () denote the number of highly susceptible cell lines out of the number of cell lines tested. Cell line deemed highly susceptible if the $EC_{50} \leq$ MOI of 1 following a 48 hr infection. MOI represents average EC_{50} [MOI] of susceptible cell lines. nd = not determined.

maintaining animals showed partial responses, delaying tumor progression by almost 2-fold compared to controls. All eight AV2-infected mice responded well to treatment with five of eight developing durable tumor regressions. In fact, no sign of tumor regrowth was evident even 7 months post-treatment. Furthermore, these mice failed to produce tumors when rechallenged with CT26 cells 7 months post-treatment, with no trace of detectable virus, perhaps indicating that host-mediated immunity to the tumor had developed. All forms of intravenous treatment were well tolerated by the mice, with no mortalities occurring and minimal signs of morbidity. Infected mice had mild to medium piloerection, mild dehydration, and some transient body weight loss following the initial treatment (Figure 4C). These symptoms were only observed after the initial infection, and all subsequent doses failed to elicit any signs of infection.

Systemic administration of AV1 and AV2 is effective against disseminated disease

CT-26 cells, when injected into the tail vein, seed tumors throughout the mouse, although predominantly within the lungs. We examined the lungs of four mice 16 days after tumor cell injection and four days after treatment with UV-inactivated virus (Figure 4D). These lungs were three times their normal mass due to their tumor burden. In contrast, tumor-bearing littermates receiving a single intravenous or intranasal dose of AV2 4 days

prior to the time of sacrifice had lungs with normal mass and few obvious tumor nodules (Figure 4D). Consistent with this result, viral gene expression could be detected within 24 hr of a single intravenous dose of GFP-expressing AV3 in all tumor nodules, with little or no detectable expression in normal lung tissue (Figure 4D; inset).

Figure 4E shows the survival plots of mice seeded with lung tumors and then treated intranasally with UV-inactivated virus, AV1 or AV2. The mean time to death (MTD) of animals treated with UV-inactivated virus was approximately 20 days. However, mice treated with either AV1 or AV2 were completely protected. This experiment demonstrates the remarkable ability of AV1 and AV2 to produce durable cures in an aggressive, disseminated, immune-competent tumor model.

Discussion

A key difference between the attenuated viruses described here and previously reported oncolytic versions of VSV is the inability of mutant M proteins of the AV viruses to block interferon production in infected cells. VSV M is a multifunctional protein required for several key viral functions including budding (Jayakar et al., 2000), virion assembly (Newcomb et al., 1982), cytopathic effect (Blondel et al., 1990), and inhibition of host gene expression (Lyles et al., 1996). The latter property has been attributed to the ability of M to block host RNA polymerase activity (Ahmed et al., 2003; Yuan et al., 2001) or to inhibit the nuclear transport of both proteins and mRNAs into and out of the host nucleus (Her et al., 1997; von Kobbe et al., 2000). The results presented here using virus infection are consistent with blocks in nuclear transport being the major mechanism by which wild-type VSV strains mitigate host antiviral response. Our analysis of infected cell transcripts provided little evidence to support a role for M protein in inhibiting host cell transcription but rather shows that VSV infection triggers an IRF-3-mediated stimulation of antiviral genes followed by an M protein-mediated block of transport of primary response transcripts from infected cell nuclei. Particularly germane to this study is the work from Dahlberg's group (Petersen et al., 2000) and others (von Kobbe et al., 2000) that has shown, by transfection studies, that M protein can associate with nuclear pore proteins and effect a block in mRNA export possibly through an association with the

Table 2B. The majority of cell lines in the NCI 60 cell panel show IFN defects

	Type I IFN defects
Leukemia	100% (6/6)*
NSC lung carcinoma	71% (5/7)
Colon carcinoma	100% (7/7)
CNS	75% (3/4)
Melanoma	85% (6/7)
Ovarian carcinoma	67% (4/6)
Renal carcinoma	75% (6/8)
Prostate	100% (2/2)
Breast	60% (3/5)
All cell lines tested	81% (42/52)

*Denotes the number of cells in each group, which were unresponsive to either IFN- α or IFN- β pre-treatment. Cell line deemed unresponsive if IFN pre-treatment was unable to significantly affect (<10-fold) the EC_{50} of cells infected with WT VSV for 48 hr.

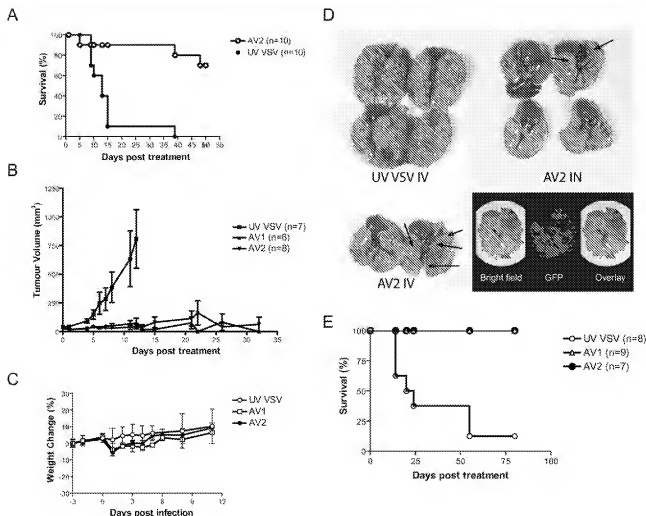


Figure 4. AV strains are efficacious in mouse tumor models

A: AV2 is effective in treating human ovarian tumor xenografts. 1×10^5 human ES-2 ovarian carcinoma cells were injected into the intraperitoneal cavity of CD-1 nude mice. Twelve days later, animals were treated by intraperitoneal injection every other day with either AV2 or UV-inactivated AV2 (1×10^6 pfu/dose; three doses total). Animals were assessed for morbidity and mortality and euthanized following the appearance of moderate ascites formation. "n" denotes number of animals per group.

B: Intravenous treatment of subcutaneous tumors. Tumors were established in the hind flank of Balb/C mice by injecting 1×10^6 CT26 cells. When tumors reached approximately 10 mm^3 , mice were treated every other day for 10 days (six doses total) with an intravenous injection of 5×10^6 pfu of the indicated virus. Control mice received six doses of 5×10^6 pfu equivalents of UV-inactivated AV2 VSV. Tumors were measured daily to calculate tumor volumes and animals were euthanized when tumors reached approximately 750 mm^3 . Error bars denote SEM.

C: Mouse weights measured daily, for each treated group, for the 3 days before treatment to day 11 post-treatment. Error bars denote SEM.

D: Treatment of disseminated lung tumors. Lung tumors were established by injecting 3×10^5 CT26 cells into the tail vein of Balb/C mice. On day 12, mice were treated as follows: UVAV2 IV = 1 dose intravenously (5×10^6 pfu equivalents), AV2 IV = 1 dose AV2 intravenously (5×10^6 pfu), AV2 IN = 1 dose of AV2 intranasally (5×10^6 pfu). Four days after treatment, all mice were sacrificed and their lungs were removed (hearts are visible for scale). Arrows indicate residual tumors. Inset: mice bearing CT-26 lung tumors were infected with AV3 GFP, and the lungs were removed and visualized as indicated.

E: Lung tumors were established as described above. On day 12, mice received 5×10^6 pfu of AV1 or AV2 by intranasal instillation every other day for 2 weeks (six doses total). "n" denotes number of mice in treatment group.

interferon-inducible cellular gene product Nup98. Indeed others have suggested that overexpression of Nup98 following interferon treatment may be sufficient to overcome an M-induced block of mRNA export. Interestingly, when we compared transcript levels in nuclear and cytoplasmic fractions following virus infection, we detected that nuclear export defects were more pronounced on some transcripts than others (D.F.S. and J.C.B., data not shown), which may reflect that the M-induced block may be specific for a subset of transcripts. It is interesting to

note that in yeast, specific mRNA export factors (Yra1 and Mex67) have been shown to be responsible for the transport of different groups of transcripts (Hieronymus and Silver, 2003). Yra1 exports transcripts depending upon their rate of transcription whereas the Mex67 export protein does not discriminate on this basis (Hieronymus and Silver, 2003). Perhaps M protein in conjunction with Nup98 is targeting a specific set of proteins involved in the regulation of export of a subset of nuclear transcripts.

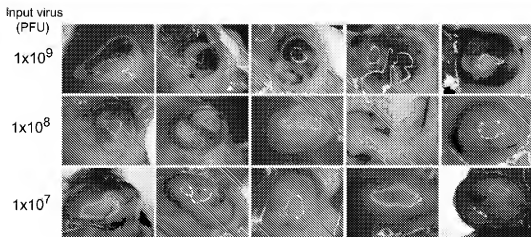


Figure 5. Threshold dose required for systemic delivery to subcutaneous tumors

Balb/c mice bearing CT26 subcutaneous tumors were treated with AV3 GFP intravenously at the indicated dose. At 24 hr, tumors were examined for fluorescence under a dissecting microscope. Tumors are shown in black and white, overlaid with fluorescent image.

It appears that host cell antiviral programs are initiated by activation of the latent transcription factors NF κ B, c-JUN/ATF2, and IRF-3. Upon viral entry into the host cell, the transcription factors c-JUN and IRF-3 are phosphorylated by JNK and a recently identified virally activated kinase (Sharma et al., 2003), while NF κ B is released from its inhibitor I κ B through the action of upstream IKK(s) (DiDonato et al., 1997). The activated transcription factors translocate to the nucleus and coordinately form an enhanosome complex at the IFN- β promoter, leading to IFN- β induction (Wathelet et al., 1998). Here we refer to this as the primary transcriptional response to virus infection. We and others (Levy, 2002; Sato et al., 1998a) postulate that a secondary transcriptional wave (or positive feedback loop; Levy, 2002) is triggered by the IFN- β -dependent induction of a variety of interferon-stimulated genes. The data presented here with wild-type M protein help to delineate the distinction between these primary and secondary transcriptional events as well as identify several novel viral response genes (*GADD34*, *PUMA*). Following infection with viruses harboring mutant M proteins, it becomes clear that autocrine stimulation of the JAK/STAT signaling pathway by IFN- β leads to the production of secondary response genes like IRF-7, which in turn are critical for the tertiary induction of IFN- α genes (Morin et al., 2002). Indeed the M protein block of secondary and tertiary transcripts can be overcome by expressing a constitutively active version of IRF-7 (from a viral promoter) even in the presence of wild-type M protein. While our cell culture studies clearly delineate the role of VSV M in blunting the positive feedback loop that is dependent upon production of IFN- β and a functional interferon receptor, WT VSV infection still is capable of inducing interferon in intact animals (albeit more slowly and to lower levels). Others have shown that in virus-infected animals, an IFN- β -independent, systemic induction of interferon can occur in certain dendritic cell subsets; however, it is the local IFN- β -dependent production of interferon that is critical in determining the magnitude and ultimate success of an interferon-mediated antiviral response (Barchet et al., 2002). We show that the amount of interferon and the timing of its production in WT VSV-infected animals are

not sufficient to protect against lethal infections. The results presented here and elsewhere (Barchet et al., 2002) are consistent with the idea that the rapid and robust local stimulation of interferon by AV strains in mice successfully attenuates virus replication in normal tissues (even of WT VSV, see Figure 1F). Our data indicates that defects in interferon signaling frequently occur during tumor evolution, with a majority of the cell lines in the NCI panel having an impaired response. Accumulating data have indicated that interferon is a multifunctional cytokine that can coordinately regulate cell growth, apoptosis, and antiviral pathways. Perhaps during tumor evolution, the selection for relentless growth and loss of apoptosis outstrips the occasional need for antiviral activity.

Kirn and colleagues argue that several factors are important in tumor killing by oncolytic virus therapeutics, including the effective delivery to multiple sites within the tumor, evasion of acquired and innate immunity, and rapid virus growth and spread (Wein et al., 2003). We have found that intravenous administration of VSV is an effective means of delivering virus to multiple sites within the tumor, and because of its broad tissue tropism and short replicative cycle, VSV can rapidly grow and spread within the tumor. These same traits, however, can be a lethal combination if virus growth in normal tissues is unchecked (Figures 1B and 1C). The attenuated viruses described here provide the best of both worlds; they grow rapidly in a broad spectrum of tumor cells but, because of their ability to trigger antiviral responses in normal cells, may be exceptionally safe to the treated animal.

One concern about the use of oncolytic virus therapeutics is the idea that a virulent strain could arise during virus propagation in a tumor. It is of interest to note, however, that it has proven impossible to date to select for VSV variants that are resistant to the antiviral effects of interferon (Novella et al., 1996), and we have shown that IFN-inducing mutants protect the host against infection with WT VSV (Figure 1F). Others have found that M mutations of the type described here cannot be complemented by mutations in other parts of M or other VSV genes (Coulon et al., 1990). In other words, only true revertants that

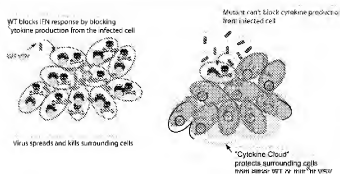


Figure 6. Model depicting how mutant VSV strains protect against virus spread.

A: WT VSV blocks IFN production from infected cells. Uninfected cells are not protected from virus spread.
B: VSV strains defective in blocking nuclear/cytoplasmic mRNA export potentially induce a "cytokine cloud" of antiviral cytokines (e.g., IFNs) protecting neighboring cells from virus spread.

convert arginine 51 back to methionine 51 can restore mutant M back to the wild-type phenotype. One of the viruses we have used in this work is a complete deletion of methionine 51, making the possibility of reversion to wild-type, even following several rounds of replication, remote. We speculate that in any population of interferon-responsive viruses where the majority of particles are potent inducers of interferon, it is unlikely that a wild-type variant could rise to dominance. The resulting "cytokine cloud" produced by infection with the IFN-inducing virus would protect the normal tissues of the host from the more virulent V strain (Figure 6). Tumor killing would, however, be unaffected, as we have shown the majority of malignancies to be defective in responding to such a "cytokine cloud." We suggest that oncolytic viruses that trigger but do not disable antiviral responses will have a significantly improved therapeutic index over viruses that lack this property.

Experimental procedures

Viruses

The Indiana serotype of VSV was used throughout this study and was propagated in Vero cells. T1026R (Desforages et al., 2001) and TP3 (Desforages et al., 2001), herein referred to as AV1 and AV2, respectively, were shown in this study and elsewhere to be IFN-inducing mutants of the HR strain of wild-type VSV Indiana (Francoeur et al., 1987). WT GFP and AV3 GFP are recombinant viruses rescued from plasmids described below. The rescue procedure has been described in detail elsewhere (Lawson et al., 1995).

Constructs and viral rescue

Creation of the constitutively active IRF-7Δ (IRF-7Δ 247–467) has been previously described elsewhere (Lin et al., 2000). IRF-7Δ 247–467 was amplified by PCR using a forward primer to the Flag epitope with an additional 5' VSV cap signal and an XhoI linker (ATCGCTCGAGAACAGATGACTA CAAAGACGATGACGACAAG), together with a specific IRF-7 reverse primer containing a VSV poly A signal and an NheI linker (ATCGGGTACGAGTTTTT TTAGGGATCCAGCTCTAGGTGG GCTGC). The PCR fragment was then cloned into the XhoI and NheI sites of the rVSV replication vector pVSV-XN2 (provided by John Rose). Recovery of rVSV has been previously described (Lawson et al., 1995).

AV3 GFP is a recombinant virus with a deletion of methionine 51 in the M protein, as well as an extra cistron-encoding green fluorescent protein (GFP) inserted between the G and L sequences. Using T4 RNA ligase, an oligonucleotide containing a consensus T7 polymerase sequence was li-

gated to the single-stranded RNA genome of the HR strain of VSV. Reverse transcription coupled PCR was used to clone the entire genome as fragments into the pBluescript II SK+ vector (Stratagene). The PCR primers were designed in such a manner as to introduce unique restriction endonuclease sites between each of the five viral cistrons. Ligation of these fragments resulted in the construction of a full-length positive sense copy of the HR VSV genome with a T7 promoter sequence at the 5' end of this anti-genome. Two overlapping oligonucleotides were synthesized to correspond to the hepatitis delta virus ribozyme sequence such that an XhoI site was introduced at the 3' terminus of the ribozyme to facilitate further cloning. These oligos were annealed and extended using Klenow to form a blunt-ended double-stranded DNA fragment. A 300 bp fragment from the 3' terminus of the anti-genome was PCR amplified and blunt ended and cloned to the ribozyme fragment. This fragment was subsequently cloned into the full-length genome construct described above via an internal AflII site in the 3' terminus of the viral genome, and the XhoI site engineered into the ribozyme fragment. Finally, the T7 terminator sequence was cloned into this vector using the flanking XhoI and Bst-III sites. This plasmid was designated pDSV1. To generate AV3VSV, we removed a XhoI/KpnI fragment spanning a region from within the P gene to within the G gene of pDSV1, replacing a similar fragment from within pXN, yielding the plasmid pXNDG. This facilitated the exchange of the M cistron from pXNDG, with one previously mutated by deleting the codon for methionine at position 51 in the amino acid sequence using directed mutagenesis (Quickchange XL; Stratagene). Subsequently, the GFP coding sequence was removed from pEGFP (Clontech) by digesting with XhoI and XbaI and ligated into XhoI and NheI sites downstream of the additional stop/start sequence in the pXNDG vector.

WT GFP VSV was constructed by inserting the coding region of GFP from pEGFP (Clontech) between the XhoI and NheI sites in the pXN vector (provided by John Rose).

IFN ELISA

Interferon-α levels were measured using a Human Interferon-Alpha ELISA kit (PBL Biomedical) per manufacturer's directions. Various cell lines were infected with either WT VSV or AV1 or AV2 VSVs at an MOI of 10. One hundred microliters of culture media was collected at 48 hr post-infection and incubated in a 96-well microtiter plate along with standards supplied by manufacturer. IFN-β production was measured at 10 hr post-infection using a human IFN-β detection kit (TFB INC; Tokyo, Japan). Samples were processed as per manufacturer's instructions and then read on a DYNEX plate reader at primary wavelength of 450 nm with a reference wavelength of 630 nm.

A variation of the above assay was used to determine the impact of IFN-β on IFN-α production. Briefly, 24-well plates of OVCA-4 cells were either mock infected or infected with WT GFP VSV or AV3 GFP VSV at an MOI of 5 for 30 min. These wells were then washed with PBS and fed with OptiMEM (Invitrogen) or OptiMEM supplemented with various concentrations of antibody as indicated. To neutralize IFN-β in the media, an anti-IFN-β antibody was used (AHC4024; Biosource International), and as a nonspecific control, an anti-HA antibody was used (sc-805; Santa Cruz). Twenty-two hours post-infection, 100 μl of media from each well was assayed for IFN-α production using a Human IFN-α ELISA (PBL Biomedical) kit as described.

Mouse serum IFN-α levels were assayed using a mouse Interferon-Alpha ELISA kit (PBL Biomedical). Balb/C females (10 weeks old; Charles River) were injected intravenously with either PBS or 1×10^6 pfu of WT GFP or AV3 GFP diluted in PBS. At the indicated times post-infection, blood was collected from the saphenous vein of each mouse into heparinized tubes and centrifuged to obtain serum. For each sample, 5 μl of serum was diluted in 95 μl of PBS and assayed as per manufacturer's instructions.

Determination of in vivo toxicity of VSV mutant viruses

Eight- to ten-week-old female mice (strains as indicated; Charles River) were divided into groups of five and infected with dilutions of virus from 1×10^{10} pfu to 1×10^1 pfu by the indicated route. Animals were monitored for weight loss, dehydration, piloerection, huddling behavior, respiratory distress, and hind limb paralysis. Mice showing moderate to severe morbidity were euthanized as per good laboratory practices prescribed by the CCAS. Lethal dose 50 values were calculated by the Karber-Spearman method.

Four-week-old Balb/C mice or Balb/C interferon-α receptor knockout

mice (IFNAR^{-/-}) (Steinhoff et al., 1995) were infected intranasally with 10⁶ pfu of either WT VSV, AV1, or AV2. Mice were monitored for signs of morbidity and were euthanized upon signs of severe respiratory distress.

Determination of *in vivo* toxicity of mixed samples of WT and mutant VSV strains

Groups of three mice were infected by intranasal instillation with either WT, AV2, or mixtures of these strains as indicated. Mice were monitored for signs of morbidity and were euthanized upon signs of severe respiratory distress or hind limb paralysis.

MTS assay

In each experiment, the test cell line was seeded into 96-well plates at 3×10^4 cells/well in growth medium (DMEM-F12-HAM + 10% FBS; Invitrogen). Following overnight incubation (37°C, 5% CO₂), media were removed by aspiration and to each well was added 20 μ l of virus-containing media (α -MEM, no serum) ranging in 10-fold increments from 3×10^6 pfu/well to 3 pfu/well or negative control media containing no virus. Each virus dose tested was done in replicates of six. After a 60 min incubation to allow virus attachment, 80 μ l of growth medium was added to each well, and the plates were incubated for another 48 hr. Cell viability was measured using the CellTiter 96 AQ_{non} MTS reagent (Promega).

To assay for IFN defects, cell lines were pretreated with either 5 units/ml of IFN- α (Schering) or IFN- β (PBL Biomedical) for 12 hr and then challenged with a range of doses of WT VSV as described above. A standard MTS assay was performed and the results compared from nonpretreated cells.

Microarray

OVCA4 cells either mock treated or infected with WT and AV4 strains were harvested in PBS, pelleted, and resuspended in 250 μ l of resuspension buffer (10 mM Tris [pH 7.4], 15 mM NaCl, 12.5 mM MgCl₂). Six hundred microliters of Lysis buffer (25 mM Tris [pH 7.4], 15 mM NaCl, 12.5 mM MgCl₂, 5% sucrose, and 1% NP-40) was added and the lysates were incubated at 4°C for 10 min with occasional vortexing. Nuclei were collected by centrifugation at 1000 \times g for 3 min. The supernatant (cytoplasmic fraction) was collected and frozen at -80°C while the pellet (nuclear fraction) was washed once with 250 μ l of lysis buffer and frozen. Total RNA was isolated from both nuclear and cytoplasmic fractions using the Qiagen RNeasy kit (as per manufacturer's instructions; Qiagen) followed by LiCl precipitation to concentrate each sample. Twenty micrograms of each RNA sample was processed according to manufacturer's standard protocol (Affymetrix) and hybridized to an Affymetrix HGA133uA chip. Each chip was scaled to 1500, normalized to the 100 normalization control genes present on each chip, then all nuclear samples were normalized to the mock nuclear sample on a per gene basis, while the cytoplasmic fractions were normalized to the corresponding mock cytoplasmic sample. Data were analyzed using GeneSpring software (SiliconGenetics).

Western blotting

OVCA4 cells were grown in RPMI (Wisent) supplemented with 10% fetal bovine serum (Wisent). 1×10^6 cells were plated in 10 cm dishes the day prior to infection. For infection, the media were removed and replaced with RPMI alone prior to the addition of 5×10^6 pfu per VSV viral strain. One hour after virus addition, media were removed and replaced with RPMI supplemented with 10% FBS for the remaining duration of the experiment. Cells were lysed in standard NP-40 lysis buffer, and 75 μ g of whole-cell extract was run on SDS-polyacrylamide gel and blotted with the following antibodies as indicated: IRF-7 (sc-9083; Santa Cruz), IRF-3 (sc-9082; Santa Cruz), ISG56 (a gift from Ganes Sen), VSV-N (polyclonal directed against the full-length Indiana N protein), and Actin (sc-8432; Santa Cruz).

Quantitative PCR of Interferon- β mRNA

Nuclear and cytoplasmic total RNA from infected or mock-infected OVCA4 cells was isolated as per manufacturer's instruction (RNeasy; Qiagen). Four micrograms of total RNA was DNase treated and reverse transcribed. Quantitative PCR was performed in triplicate to amplify IFN- β and HPRT targets from each using Roche LightCycler technology (Roche Diagnostics). Crossing points were converted to absolute quantities based on standard curves generated for each target amplicon. IFN- β signal was subsequently normalized to HPRT as HPRT levels are unchanged during the course of these

infections (data not shown). Primers used to amplify IFN- β were sense 5'-TTGTGCTCTCTCACTACAGC-3', antisense 5'-CTGTAGTCTGTTAATGAAG-3' and HPRT primers were sense 5'-TGACACTGGCAAAACAA TGA-3', antisense 5'-GGTCTTTTACCAGCAAGCT-3'.

RT-PCR of interferon- α and interferon-stimulated genes

A549 cells cultured in F12K medium supplemented with 10% FBS were infected with WT or AV strains (MOI 10). RNA was extracted 4 hr post-infection using Trizol (Invitrogen) according to the manufacturer's instructions. One microgram of RNA was reverse transcribed with Oligo dT primers and 5% of RT was used as template in a Taq PCR. Primers used were as follows: Mx forward primer 5'-TTTGTGTTTTCGGAAGTGGAC-3' and reverse primer 5'-TTTCTTCAGTTTCAGCAGCAGC-3'; VSV N forward primer 5'-ATGTCTGTACAGTCAAGGAATC-3' and reverse primer 5'-TCATTGTG CAAATTCGTACTAGCATA-3'; RANTES forward primer 5'-TACACAGGTG CCAAGTGTCTCAACCCAG-3' and reverse primer 5'-GTCTCGAAGCTCT GACCTCAAGTGATCC-3'; β -actin forward primer 5'-ACAATGAGCTGC TGGTGGCT-3' and reverse primer 5'-GATGGGCACAGTGTGGTGA-3'.

Ovarian xenograft cancer model in athymic mice

Approximately 1×10^6 ES-2 human ovarian carcinoma cells were injected into the peritoneal cavity of CD-1 athymic mice (Charles River). Ascites development is generally observed by day 15 after cell injection. On days 12, 14, and 16, mice were treated with 1×10^6 AV2 virus or 1×10^6 pfu equivalent of UV-inactivated AV2 VSV by intraperitoneal injection. Mice were monitored for morbidity and euthanized upon development of ascites.

Subcutaneous tumor model

To establish subcutaneous tumors, 8- to 10-week-old Balb/C female mice (Charles River) were shaved on the right flank and injected with 1×10^6 CT26 colon carcinoma cells (Kashtan et al., 1992) syngeneic for Balb/C mice. These tumors were allowed to develop until they reached approximately 10 mm³, at which time virus treatments were initiated. Groups of animals received 1 of 6 doses of the indicated virus every other day for 2 weeks. Each dose of 5×10^6 pfu was administered by tail vein injection. Tumors were measured daily and volumes calculated using the formula $1/2(L \times W \times H)$. Mice were weighed daily and monitored for weight loss, dehydration, piloerection, huddling behavior, respiratory distress, and hind limb paralysis. Animals were euthanized when their tumor burden reached end point (750 mm³).

Lung model

Lung tumors were established in 8- to 10-week-old female Balb/C mice (Charles River) by tail vein injection of 3×10^6 CT26 cells (Specht et al., 1997). On days 10, 12, 14, 17, 19, and 21, groups of mice received 5×10^6 pfu of the indicated virus by intranasal instillation as described elsewhere (Stojdl et al., 2000a). Mice were weighed daily and monitored for weight loss, dehydration, piloerection, huddling behavior, respiratory distress, and hind limb paralysis. Animals were euthanized at the onset of respiratory distress and their lungs examined to confirm tumor development.

Visualization of GFP-expressing VSV strains *in vivo*

Female Balb/C mice (Charles River) were injected with 3×10^6 CT26 cells via the vein to initiate pulmonary metastases. On day 17, mice were injected intravenously with 2.5×10^6 pfu AV3 GFP VSV. Hind limb tumors were seeded by subcutaneous injection with 3×10^6 CT26 cells. When tumors reached a volume of approximately 400 mm³, mice were injected intravenously with 2.5×10^6 pfu of AV3 GFP VSV. At the indicated times, mice were euthanized and tumors examined using a Leica MZFLII microscope with a standard GFP filter set. Pictures were captured with a Nikon Coolpix 100 camera. Overlayed images were generated using Adobe Photoshop 7.0.

Acknowledgments

B.D.L. is a recipient of a fellowship from the National Cancer Institute of Canada (NCIC). This work was funded by grants from the NCIC and the Canadian Institutes of Health Research awarded to J.C.B. Funding for portions of this work was also provided by Wellstar Biologics Corporation. J.C.B. is Senior Scientist at Cancer Care Ontario. We thank John Rose for plasmids required to produce recombinant VSVs and Ken Garson for critical reading of the manuscript.

Received: April 15, 2003

Revised: July 31, 2003

Published: October 20, 2003

References

- Ahmed, M., McKenzie, M.O., Puckett, S., Hohnacki, M., Poliquin, L., and Lyles, D.S. (2003). Ability of the matrix protein of vesicular stomatitis virus to suppress beta interferon gene expression is genetically correlated with the inhibition of host RNA and protein synthesis. *J. Virol.* 77, 4646–4657.
- Balachandran, S., and Barber, G.N. (2000). Vesicular stomatitis virus (VSV) therapy of tumors. *IUBMB Life* 50, 135–138.
- Balachandran, S., Roberts, P.C., Brown, L.E., Truong, H., Pattnaik, A.K., Archer, D.R., and Barber, G.N. (2000). Essential role for the dsRNA-dependent protein kinase PKR in innate immunity to viral infection. *Immunity* 13, 129–141.
- Barchet, W., Cella, M., Odermatt, B., Asselin-Paturel, C., Colonna, M., and Kalinke, U. (2002). Virus-induced interferon alpha production by a dendritic cell subset in the absence of feedback signaling in vivo. *J. Exp. Med.* 195, 507–516.
- Bell, J.C., Garson, K.A., Lichty, B.D., and Stojdl, D.F. (2002). Oncolytic viruses: programmable tumour hunters. *Curr. Gene Ther.* 2, 243–254.
- Bello, M.J., de Campos, J.M., Kusak, M.E., Vaquero, J., Sarasa, J.L., Pestana, A., and Rey, J.A. (1994). Molecular analysis of genomic abnormalities in human gliomas. *Cancer Genet. Cytogenet.* 73, 122–129.
- Blondel, D., Hamison, G.G., and Schubert, M. (1990). Role of matrix protein in cytopathogenesis of vesicular stomatitis virus. *J. Virol.* 64, 1716–1725.
- Coulon, P., Deutsch, V., Lafay, F., Martinet-Edelstein, C., Wyers, F., Herman, R.C., and Flamand, A. (1990). Genetic evidence for multiple functions of the matrix protein of vesicular stomatitis virus. *J. Gen. Virol.* 71, 991–996.
- Desforges, M., Charron, J., Berard, S., Beausoleil, S., Stojdl, D.F., Despars, G., Laverdiere, B., Bell, J.C., Talbot, P.J., Stanners, C.P., and Poliquin, L. (2001). Different host-cell shutoff strategies related to the matrix protein lead to persistence of vesicular stomatitis virus mutants on fibroblast cells. *Virus Res.* 76, 87–102.
- DiDonato, J.A., Hayakawa, M., Rothwarf, D.M., Zandi, E., and Karin, M. (1997). A cytokine-responsive I κ B kinase that activates the transcription factor NF- κ B. *Nature* 388, 548–554.
- Ferran, M.C., and Lucas-Lenard, J.M. (1997). The vesicular stomatitis virus matrix protein inhibits transcription from the human beta interferon promoter. *J. Virol.* 71, 371–377.
- Francoeur, A.M., Poliquin, L., and Stanners, C.P. (1987). The isolation of interferon-inducing mutants of vesicular stomatitis virus with altered viral P function for the inhibition of total protein synthesis. *Virology* 160, 236–245.
- Genin, P., Algarte, M., Roof, P., Lin, R., and Hiscott, J. (2000). Regulation of RANTES chemokine gene expression requires cooperatively between NF- κ B and IFN-regulatory factor transcription factors. *J. Immunol.* 164, 5352–5361.
- Gromeier, M., and Wimmer, E. (2001). Viruses for the treatment of malignant glioma. *Curr. Opin. Mol. Ther.* 3, 503–508.
- Hawkins, L.K., Lemoine, N.R., and Kim, D. (2002). Oncolytic biotherapy: a novel therapeutic platform. *Lancet Oncol.* 3, 17–26.
- Her, L.S., Lund, E., and Dahlberg, J.E. (1997). Inhibition of Ran guanosine triphosphatase-dependent nuclear transport by the matrix protein of vesicular stomatitis virus. *Science* 276, 1845–1848.
- Hieronymus, H., and Silver, P.A. (2003). Genome-wide analysis of RNA-protein interactions illustrates specificity of the mRNA export machinery. *Nat. Genet.* 33, 155–161.
- Huneycutt, B.S., Bi, Z., Aoki, C.J., and Reiss, C.S. (1993). Central neuropathogenesis of vesicular stomatitis virus infection of immunodeficient mice. *J. Virol.* 67, 6698–6706.
- Ikeda, K., Wakimoto, H., Ichikawa, T., Jhung, S., Hochberg, F.H., Louis, D.N., and Chiochia, E.A. (2000). Complement depletion facilitates the infection of multiple brain tumors by an intravascular, replication-conditional herpes simplex virus mutant. *J. Virol.* 74, 4765–4775.
- Jayakar, H.R., Murli, K.G., and Whitt, M.A. (2000). Mutations in the PPPY motif of vesicular stomatitis virus matrix protein reduce virus budding by inhibiting a late step in virion release. *J. Virol.* 74, 9818–9827.
- Kashan, H., Rabau, M., Mullen, J.B., Wong, A.H., Roder, J.C., Shpitz, B., Stern, H.S., and Gallinger, S. (1992). Intra-rectal injection of tumour cells: a novel animal model of rectal cancer. *Surg. Oncol.* 1, 251–256.
- Krutz, F.A., and Curiel, D.T. (2002). Toward a new generation of conditionally replicating adenoviruses: pairing tumor selectivity with maximal oncolysis. *Hum. Gene Ther.* 13, 485–495.
- Lawson, N.D., Stillman, E.A., Whitt, M.A., and Rose, J.K. (1995). Recombinant vesicular stomatitis viruses from DNA. *Proc. Natl. Acad. Sci. USA* 92, 4477–4481.
- Levy, D.E. (2002). Whence interferon? Variety in the production of interferon in response to viral infection. *J. Exp. Med.* 195, F15–F18.
- Lin, R., Mamane, Y., and Hiscott, J. (2000). Multiple regulatory domains control IRF-7 activity in response to virus infection. *J. Biol. Chem.* 275, 34320–34327.
- Linge, C., Gewert, D., Rossmann, C., Bishop, J.A., and Crowe, J.S. (1995). Interferon system defects in human malignant melanoma. *Cancer Res.* 55, 4099–4104.
- Lu, R., Au, W.C., Yeow, W.S., Hageman, N., and Pitha, P.M. (2000). Regulation of the promoter activity of interferon regulatory factor-7 gene. Activation by interferon and silencing by hypermethylation. *J. Biol. Chem.* 275, 31805–31812.
- Lyles, D.S., McKenzie, M.O., Ahmed, M., and Woolwine, S.C. (1996). Potency of wild-type and temperature-sensitive vesicular stomatitis virus matrix protein in the inhibition of host-directed gene expression. *Virology* 225, 172–180.
- Matin, S.F., Rackley, R.R., Sadhukhan, P.C., Kim, M.S., Novick, A.C., and Bandyopadhyay, S.K. (2001). Impaired alpha-interferon signaling in transitional cell carcinoma: lack of p48 expression in 5637 cells. *Cancer Res.* 61, 2261–2266.
- Morin, P., Braganca, J., Bandu, M.T., Lin, R., Hiscott, J., Doly, J., and Civas, A. (2002). Preferential binding sites for interferon regulatory factors 3 and 7 involved in interferon- α gene transcription. *J. Mol. Biol.* 316, 1009–1022.
- Nakaya, T., Sato, M., Hata, N., Asagiri, M., Suemori, H., Noguchi, S., Tanaka, N., and Taniguchi, T. (2001). Gene induction pathways mediated by distinct IRFs during viral infection. *Biochem. Biophys. Res. Commun.* 283, 1150–1156.
- Newcomb, W.W., Tobin, G.J., McGowan, J.J., and Brown, J.C. (1982). In vitro reassembly of vesicular stomatitis virus skeletons. *J. Virol.* 41, 1055–1062.
- Norman, K.L., Farassati, F., and Lee, P.W. (2001). Oncolytic viruses and cancer therapy. *Cytokine Growth Factor Rev.* 12, 271–282.
- Novella, I.S., Clinis, M., Elena, S.F., Kohn, J., Moya, A., Domingo, E., and Holland, J.J. (1996). Large population passages of vesicular stomatitis virus in interferon-treated cells select variants of only limited resistance. *J. Virol.* 70, 6414–6417.
- Petersen, J.M., Her, L.S., Varvel, V., Lund, E., and Dahlberg, J.E. (2000). The matrix protein of vesicular stomatitis virus inhibits nucleocytoplasmic transport when it is in the nucleus and associated with nuclear pore complexes. *Mol. Cell. Biol.* 20, 8590–8601.
- Sato, M., Hata, N., Asagiri, M., Nakaya, T., Taniguchi, T., and Tanaka, N. (1998a). Positive feedback regulation of type I IFN genes by the IFN-inducible transcription factor IRF-7. *FEBS Lett.* 441, 106–110.
- Sato, M., Tanaka, N., Hata, N., Oda, E., and Taniguchi, T. (1998b). Involvement of the IRF family transcription factor IRF-3 in virus-induced activation of the IFN- β gene. *FEBS Lett.* 425, 112–116.
- Sharma, S., tenOver, B.R., Grandvaux, N., Zhou, G.P., Lin, R., and Hiscott, J. (2003). Triggering the interferon antiviral response through an IKK-related pathway. *Science* 300, 1148–1151.

- Specht, J.M., Wang, G., Do, M.T., Lam, J.S., Royal, R.E., Reeves, M.E., Rosenberg, S.A., and Hwu, P. (1997). Dendritic cells retrovirally transduced with a model antigen gene are therapeutically effective against established pulmonary metastases. *J. Exp. Med.* 186, 1213-1221.
- Steinhoff, U., Muller, U., Schertler, A., Hengartner, H., Aguet, M., and Zinkernagel, R.M. (1995). Antiviral protection by vesicular stomatitis virus-specific antibodies in alpha/beta interferon receptor-deficient mice. *J. Virol.* 69, 2153-2158.
- Stojdl, D.F., Abraham, N., Knowles, S., Marius, R., Brasey, A., Lichty, B.D., Brown, E.G., Sonenberg, N., and Bell, J.C. (2000a). The murine double-stranded RNA-dependent protein kinase PKR is required for resistance to vesicular stomatitis virus. *J. Virol.* 74, 9580-9585.
- Stojdl, D.F., Lichty, B., Knowles, S., Marius, R., Atkins, H., Sonenberg, N., and Bell, J.C. (2000b). Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus. *Nat. Med.* 6, 821-825.
- Sun, W.H., Pabon, C., Alsayed, Y., Huang, P.P., Jandeska, S., Uddin, S., Platanius, L.C., and Rosen, S.T. (1998). Interferon-alpha resistance in a cutaneous T-cell lymphoma cell line is associated with lack of STAT1 expression. *Blood* 91, 570-576.
- Terstegen, L., Gatsios, P., Ludwig, S., Pleschka, S., Jahnke-Dechent, W., Heinrich, P.C., and Graeve, L. (2001). The vesicular stomatitis virus matrix protein inhibits glycoprotein 130-dependent STAT activation. *J. Immunol.* 167, 5209-5216.
- von Kobbe, C., van Deursen, J.M., Rodrigues, J.P., Sitterlin, D., Bachi, A., Wu, X., Wilm, M., Carmo-Fonseca, M., and Izaurralde, E. (2000). Vesicular stomatitis virus matrix protein inhibits host cell gene expression by targeting the nucleoporin Nup98. *Mol. Cell* 6, 1243-1252.
- Wakimoto, H., Ikeda, K., Abe, T., Ichikawa, T., Hochberg, F.H., Ezekowitz, R.A., Pasternack, M.S., and Chiocca, E.A. (2002). The complement response against an oncolytic virus is species-specific in its activation pathways. *Mol. Ther.* 5, 275-282.
- Wakimoto, H., Johnson, P.R., Knipe, D.M., and Chiocca, E.A. (2003). Effects of innate immunity on herpes simplex virus and its ability to kill tumor cells. *Gene Ther.* 10, 983-990.
- Wathelet, M.G., Lin, C.H., Parekh, B.S., Ronco, L.V., Howley, P.M., and Maniatis, T. (1998). Virus infection induces the assembly of coordinately activated transcription factors on the IFN-beta enhancer in vivo. *Mol. Cell* 1, 507-518.
- Wein, L.M., Wu, J.T., and Kim, D.H. (2003). Validation and analysis of a mathematical model of a replication-competent oncolytic virus for cancer treatment: implications for virus design and delivery. *Cancer Res.* 63, 1317-1324.
- Wong, L.H., Krauer, K.G., Hatzinisiriou, I., Estcourt, M.J., Hersey, P., Tam, N.D., Edmondson, S., Devenish, R.J., and Ralph, S.J. (1997). Interferon-resistant human melanoma cells are deficient in ISGF3 components, STAT1, STAT2, and p48-ISGF3gamma. *J. Biol. Chem.* 272, 28779-28785.
- Yoon, S.K., Armentano, D., Wands, J.R., and Mohr, L. (2001). Adenovirus-mediated gene transfer to orthotopic hepatocellular carcinomas in athymic nude mice. *Cancer Gene Ther.* 8, 573-579.
- Yuan, H., Puckett, S., and Lyles, D.S. (2001). Inhibition of host transcription by vesicular stomatitis virus involves a novel mechanism that is independent of phosphorylation of TATA-binding protein (TBP) or association of TBP with TBP-associated factor subunits. *J. Virol.* 75, 4453-4458.

Clinical Predictive Value of the *in Vitro* Cell Line, Human Xenograft, and Mouse Allograft Preclinical Cancer Models¹

Theodora Voskoglou-Nomikos,² Joseph L. Pater, and Lesley Seymour

National Cancer Institute of Canada Clinical Trials Group, Cancer Clinical Trials Division, Cancer Research Institute, Queen's University, Kingston, Ontario, K7L 3N6 Canada

ABSTRACT

Purpose: We looked at the value of three preclinical cancer models, the *in vitro* human cell line, the human xenograft, and the murine allograft, to examine whether they are reliable in predicting clinical utility.

Experimental Design: Thirty-one cytotoxic cancer drugs were selected. Literature was searched for drug activity in Phase II trials, human xenograft, and mouse allografts in breast, non-small cell lung, ovary, and colon cancers. Data from the National Cancer Institute Human Tumor Cell Line Screen were used to calculate drug *in vitro* preclinical activity for each cancer type. Phase II activity *versus* preclinical activity scatter plot and correlation analysis was conducted for each model, by tumor type (disease-oriented approach), using one tumor type as a predictor of overall activity in the other three tumor types combined (compound-oriented approach) and for all four tumor types together.

Results: The *in vitro* cell line model was predictive for non-small cell lung cancer under the disease-oriented approach, for breast and ovarian cancers under the compound-oriented approach, and for all four tumor types together. The mouse allograft model was not predictive. The human xenograft model was not predictive for breast or colon cancers, but was predictive for non-small cell lung and ovarian cancers when panels of xenografts were used.

Conclusions: These results suggest that under the right framework and when panels are used, the *in vitro* cell line and human xenograft models may be useful in predicting the Phase II clinical trial performance of cancer drugs. Murine

allograft models, as used in this analysis, appear of limited utility.

INTRODUCTION

Both basic science studies and clinical trials are essential components of the cancer drug discovery process. Potential therapeutics found to be significantly better than no treatment or standard therapies (*i.e.*, active) in preclinical laboratory cancer models or compounds with novel chemotypes and equivalent effectiveness to standard treatments are advanced to confirmatory testing in early (Phase I and II) clinical trials. Considering that RR³ is a reasonable surrogate end point for survival (required but not sufficient), a favorable RR in Phase II trials advances a drug into additional clinical testing and is considered a prerequisite of drug success in the clinic.

Advancing of a candidate drug from preclinical testing in the laboratory to testing in Phase II clinical trials is based on the assumption that drug activity in cancer models translates into at least some efficacy in human patients, *i.e.*, that cancer laboratory models are clinically predictive. In addition, the relevance of tumor type-specific preclinical results for the corresponding human cancers in the clinic can be viewed through two different approaches: compound-oriented, where a drug is assumed to have potential activity against all human tumor types if it is effective against a single test tumor type, and disease-oriented, where a drug with preclinical activity in a single tumor type would only be expected to be effective in the same tumor type in patients.

Although widely adopted, the above-mentioned assumption and approaches have not been confirmed by studies to date. In addition, all studies aimed to examine the clinical predictive value of laboratory cancer models inevitably suffer from inherent bias because compounds with no activity in preclinical models are generally not advanced to clinical trials.

This work was undertaken to examine the clinical predictive value of three preclinical cancer models that have found wide use: the human *in vitro* cell line; the mouse allograft; and the human xenograft. In these models, tumor volume or life span (*in vivo* mouse models) or cell growth (*in vitro* cell lines) is compared between the treatment group receiving the new drug and a control group (active or inactive control).

The use of preclinical cancer models for selection of potential cancer therapeutics was pioneered by the NCI in the United States in the mid-1950s. The screening strategies used until 1990 were essentially compound oriented and involved a

Received 3/26/03; revised 6/3/03; accepted 6/4/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹Supported in part by the National Cancer Institute of Canada, Clinical Trials Group. Presented in part at the 2002 Annual Meeting of the American Society of Clinical Oncology (Abstract 360).

²To whom requests for reprints should be addressed, at National Cancer Institute of Canada Clinical Trials Group, Cancer Clinical Trials Division, Cancer Research Institute, Queen's University, 10 Stuart Street, Kingston, Ontario, K7L 3N6 Canada. Phone: (613) 533-6430; Fax: (613) 533-2941; E-mail: dnomikos@ctg.queensu.ca.

³The abbreviations used are: RR, response rate; NCI, National Cancer Institute; NSCLC, non-small cell lung cancer; NSC, National Science Center; T/C%, treated over control tumor volume ratio.

small number of predominantly murine allograft tumors, with emphasis on leukemia (1-7). Several studies from the NCI and others demonstrated that this approach had low clinical predictive value for activity in Phase II trials (5-9) and yielded compounds with selective activity toward human leukemias and lymphomas (10-12). Thus, in 1990, the NCI introduced a disease-oriented *in vitro* Human Tumor Cell Line Screen comprised of 60 cell lines from the most common adult tumors (13-17). The screen was designed so that each tumor type was represented by a panel of cell lines, selected on the basis of different subhistological features, and common drug resistance profiles. It was hoped that this screen would help identify drug leads with high potency and/or selective activity against particular tumor types.

Recently, the NCI examined the correlation between drug activity in Phase II clinical trials and preclinical activity in cancer models (18). Important findings were: (a) with the exception of NSCLC, preclinical activity in human xenografts of a particular tumor type did not correlate significantly with Phase II activity in the same type of tumor, (b) with the exception of breast and colon histologies, human xenografts did not significantly predict Phase II clinical activity in other cancers types; and (c) compounds that were active in at least one-third of all tested human xenografts were likely to have at least some activity in Phase II clinical trials.

Studies examining the clinical predictive value of preclinical cancer models outside the scope of the NCI screening programs have focused on the human xenograft model and have looked predominantly into same-tumor correlations (disease-oriented approach). These studies have produced both positive (the model was found clinically predictive) and negative (the model was found to have no clinical predictive value) results in various tumor types (19-27).

Two major criticisms can be made on the overall body of literature concerning the clinical predictive value of preclinical cancer models. First, the vast majority of studies to date, both within and outside the NCI, have based their conclusions on the observation of trends rather than the use of statistical methods. Second, all studies conducted previously have used dichotomous definitions of preclinical and/or clinical activity based on largely invalidated cutoff values of measures of activity: a 20% RR in Phase II clinical trials and (most commonly) a 42% T/C% in human xenografts and mouse allografts.

In addition, two important questions have not been addressed at all by previous studies: the clinical predictive value of the *in vitro* cell line model and the relative clinical usefulness of the different preclinical cancer models in use today (*i.e.*, how different models compare with each other in terms of their ability to identify clinically effective drugs).

Thus, we conducted a study comparing the clinical (Phase II) predictive value of three widely used preclinical laboratory cancer models, the *in vitro* human cell line, the mouse allograft, and the human xenograft. We used quantitative measures of both clinical and preclinical activity and statistical methods. We considered three relevant questions: (a) the clinical predictive value of the three models within the same tumor type (disease-oriented approach); (b) the clinical predictive value of the three models when one preclinical tumor type is used as a predictor of overall clinical activity in all other tumor types (compound-

oriented approach); and (c) the clinical predictive value of the three models when overall preclinical and clinical activity in all tumor types combined is considered.

MATERIALS AND METHODS

Study Design

A retrospective, literature-based study was conducted. Data were retrieved from studies published between 1985 and 2000. This period was chosen as one when all three preclinical cancer models of interest to this study were in use and because it was long enough and close enough to the present as to afford data on a relatively large number of recently developed drugs.

The data search was restricted to four of the most common and commonly studied solid tumor types, breast, colorectal, ovarian, and non-small cell lung cancers, to ensure that sufficient data would be available.

The Medline and CancerLit databases were used for the collection of published data. In an attempt to minimize publication bias, both paper publications (peer reviewed) and meeting abstracts (nonpeer reviewed) were used as sources of information. If published data were not available for identified drugs, manufacturers were contacted for unpublished data.

Selection of Drugs

Drugs were identified by searching the Medline and CancerLit databases for compounds that had undergone single agent Phase I clinical trial testing either in 1991 or 1992. Agents with novel targets such as signal transduction or angiogenesis modulators were not included.

This Phase I-based approach to agent identification was used to ensure selection of agents developed within the study time frame of 1985-2000: agents with a published Phase I clinical trial in 1991 or 1992 were expected to have been through preclinical testing between 1985 and 1990 and to have undergone Phase II clinical evaluation by the year 2000. In addition, this approach was adopted to minimize publication bias: publication of Phase I trials is generally less dependent on the observation of favorable tumor responses than publication of Phase II trials or of preclinical cancer model experiments.

Data Collection and Drug Activity

Phase II Clinical Trials. Phase II clinical trials for each drug were identified by searching the Medline and CancerLit databases for scientific papers, reviews, or meeting abstracts. Duplicate publications were discarded. For trials with only abstract information, an additional search by author and/or institution name was conducted in Medline or CancerLit. Scientific papers were used in preference to abstracts, where possible.

Two restrictions were applied. The first was a geographic restriction: to ensure uniform methodology in trial conduct and RR assessment, only Phase II trials conducted in the Americas, Western Europe and Australia were included in the analysis. The second restriction referred to the treatment population and aimed to ensure that uniformly responsive populations of patients would be considered. For breast and ovarian cancer, only Phase II trials that included patients who had received prior chemotherapy for metastatic disease were used, whereas for

NSCLC and colon cancers, the Phase II trials selected included patients who had received no prior chemotherapy.

For each individual Phase II trial the following information was collected: disease site; previous chemotherapy; disease stage; number of patients entered; eligible; evaluable and evaluable for response; number of complete and partial responses; and criteria used for response (standard WHO *versus* other). Trials had to have enrolled a minimum of 14 patients, at least 12 of whom must have been evaluable for response. Completed Phase II trials for which >20% of entered patients were listed as evaluable for response were considered methodologically unacceptable and were not used. For trials in progress at the time of reporting (meeting abstract format only), the available data were used even if they represented <80% of the enrolled patients, provided that they met the 14-patient criterion. If a trial publication did not specify the previous chemotherapy treatment status of patients, it was not used. Information from Phase I-II trials was used only when the Phase I and II components of the trial were separately conducted and reported. Phase II information was collected regardless of drug dose and route of administration.

For a given drug, in a given cancer type, the activity in a single Phase II clinical trial was recorded as the RR: the number of partial and complete tumor responses over the total number of patients evaluable for response. The number of evaluable rather than eligible patients was used to accommodate information from trials for which final results were not available. In the very few cases where the number of patients evaluable for response was not provided, the number of evaluable patients, the number of eligible patients, or the number of patients entered in the trial (whichever was provided by the investigators) in that priority order was used.

To obtain a drug's overall clinical activity in multiple Phase II trials of patients with the same tumor type, all responses and the collective number of patients evaluable for response were pooled from individual trials to calculate an overall RR. Finally, to get the Phase II activity for any three or four cancer types combined, the individual tumor RRs were averaged.

Human Xenografts and Mouse Allografts. The search strategy for mouse cancer model data were similar to the Phase II process. The only exclusion in this case were results obtained with mouse tumors that were engineered to have special characteristics such as, for example, overexpression of proteins conferring drug resistance.

For each murine allograft or human xenograft, numerical value(s) of activity for drugs of interest was retrieved only if expressed as the treated over control tumor volume ratio (T/C%) or the tumor volume growth inhibition ratio (GI%); and T/C% = $100\% - \text{GI}\%$ in the literature sources. In addition, only T/C% values calculated by the formula $\text{T/C}\% = [(RV_{\text{treated}})/(RV_{\text{control}})] \times 100\%$ were collected (where RV = relative volume), whereas T/C% values defined for regressions $\text{T/C}\% = [(RV_{\text{treated}}(0) - RV_{\text{treated}}(t))/RV_{\text{treated}}(0)] \times 100\%$ were excluded to ensure uniform calculation methods. If the T/C% was not provided but a relative tumor growth curve was given as a figure in a publication, the numerical values for the treatment and control groups provided in this graph were used to calculate the T/C%. Activity reported as all mice cured or 100% complete responses was considered equivalent to and recorded as a T/C%

= 0. If no exact T/C% value was given but an interval of values was provided instead (i.e., T/C% > 42), a T/C% equal to the interval midpoint value (i.e., a T/C% = 71) was assigned. Finally, where preclinical activity was reported as GI%, it was converted to T/C% by the formula $\text{T/C}\% = 100\% - \text{GI}\%$. The activity value for the most effective, nontoxic dose in each schedule was recorded.

Single tumor type preclinical activity of each drug in the murine allograft or human xenograft models was defined as the mean T/C% value from all tested allografts/xenografts of that tumor type. Where the same laboratory had tested a single xenograft/allograft with multiple schedules of the same drug and/or where the same xenograft/allograft had been tested with the same drug by more than one laboratories, T/C% values for a single tumor were obtained by first averaging the same laboratory T/C% values and then the same xenograft T/C% values.

Overall preclinical activity in xenografts/allografts for all four tumor types together was expressed as the average of single tumor mean T/C% values.

In Vitro Human Tumor Cell Lines. The publicly available data from the NCI's Human Tumor Cell Line Screen was used as the information source for the *in vitro* tumor cell line model. Information from the NCI *in vitro* Human Tumor Cell Line Screen was favored because it was a readily available, well-defined, comprehensive, validated, and extensive single source of data. Another important reason was that as an exploratory literature search showed, there was such a wide variation between different investigators in the types of assays used and the nature of cell lines tested that it would have been impossible to comprehensively combine published data from various laboratories.

Acquisition of NCI Human Tumor Cell Line Screen data were done through the internet.⁴ Information for each drug was obtained through its NCI code number or NSC number. Such numbers, where available, were identified either from the literature or from a cross-reference of compound names and NSC numbers in the NCI database (also available on the NCI web site).⁴

Testing of compounds in the NCI *in vitro* Human Tumor Cell Line Screen has been described previously (17). Briefly, growth inhibition in cell lines is measured by the GI_{50} , defined as the drug concentration that causes a 50% reduction in cell number in test plates relative to control plates. For every drug entering the screen, a concentration range comprised of five, 10-fold dilutions is tested in each of a group of 60–80 cell lines. The optical densities between treated and control plates, as resulting from the sulforhodamine B assay, are used to construct a dose-response curve for each cell line in the screen, leading to the calculation of a GI_{50} in every case by interpolation. In the case of compounds with low (i.e., the highest concentration tested causes <50% growth inhibition) or high (i.e., the lowest concentration tested causes >50% growth inhibition) potency where interpolation is not possible, the highest and lowest concentrations, respectively, in the tested drug concentration

⁴ Internet address: http://www.dtp.nci.nih.gov/docs/cancer/searches/cancer_open_compounds.html.

range are recorded as the approximated GI_{50} s. GI_{50} s are then converted to their Log_{10} values and the overall mean $Log_{10}GI_{50}$ across all cell lines in the screen is calculated. Finally, the results are displayed by a bar graph called the mean graph (28). This graph lists all of the cell lines and their corresponding $Log_{10}GI_{50}$ s and relates the magnitude of every individual cell line $Log_{10}GI_{50}$ to the mean $Log_{10}GI_{50}$ across all of the cell lines by a bar to the right (more sensitive than average) or to the left (less sensitive than average) of a vertical line. The experiment is repeated several times for each concentration range. In cases where mean graphs are based on mostly approximated GI_{50} s, other higher or lower concentration ranges of the drug (again made of five, 10-fold dilutions) are also tested. Thus, for each compound tested in the NCI *in vitro* Human Tumor Cell Line Screen, multiple GI_{50} mean graphs (one for each concentration range tested) based on multiple experiments each and with a different content of approximated *versus* calculated (by interpolation) GI_{50} s may exist in the NCI database.

We obtained all of the available GI_{50} mean graph information from the NCI web site for all drugs in our list of compounds with known NSC numbers.⁴ For every drug, we recorded the number of concentration ranges tested in the NCI *in vitro* Human Tumor Cell Line Screen, the number of experimental repetitions conducted for each concentration range, and, finally, the number of approximated $Log_{10}GI_{50}$ s in each mean graph.

The drug concentration range that produced the mean graph with the smallest number of approximated $Log_{10}GI_{50}$ s was used for scoring a drug's activity in the NCI *in vitro* Human Tumor Cell Line Screen, unless a different concentration range existed, with a number of approximated $Log_{10}GI_{50}$ s varying <10% from the first but for which more experiments were done.

Preclinical activity in the NCI *in vitro* Human Tumor Cell Line Screen was scored in two different ways: by the mean $Log_{10}GI_{50}$ and by what was termed the activity fraction. For a given drug, in a given tumor type, the mean $Log_{10}GI_{50}$ was computed by averaging the $Log_{10}GI_{50}$ s from all of the cell lines of that tumor type in the mean graph corresponding to the most appropriate concentration range. The activity fraction was arbitrarily defined as the number of cell lines of a given tumor type in which the individual $Log_{10}GI_{50}$ s were more sensitive to the drug than the average $Log_{10}GI_{50}$ (for all cell lines of all cell types) in the mean graph over the total number of cell lines tested from that tumor type. The activity fraction was also calculated from the mean graph corresponding to the most appropriate concentration range. Overall mean $Log_{10}GI_{50}$ s or activity fractions for all four cancer types combined were calculated by averaging the single tumor values.

Statistical Analysis

For each preclinical cancer model, 9 Phase II *versus* preclinical activity relationships were examined for a total of 27: relationships by tumor type (disease-oriented approach, 4 relationships/model), predictive ability of one tumor type for the other three tumor types combined (compound-oriented approach, 4 relationships/model), and general predictive ability for all four tumor types combined (1 relationship/model).

Relationships were first examined descriptively with the construction of various Phase II overall activity *versus* preclinical

Table 1. Drugs selected for data collection. NSC numbers are shown, where available

Drug	NSC number
Taxotere	628503
Paclitaxel	125973
Topotecan	609699
Irinotecan	
Rhizoxin	332598
Gemcitabine	
Fazarabine	281272
Teniposide	122819
Menogaril	269148
Fosquidone	D611615
Ethamivirin	369327
Amonafide	308847
Didemnin B	352319
Suramin	
Raltitrexed	639186
Flavone acetic acid	347512
Epirubicin	256942
CI-921	343499
Trimetrexate	352122
Multitargeted antifol	
Vincorelbine	
Piritrexim	351521
Fosmustine	
CI-930	
Chloroquinotaxine sulfonamide	339004
Imofosine	
CI-941	
Tiazofurin	286193
Pyrazine diazohydroxide	361456
Tallimustine	
Crisnatol	

ical activity scatter plots (Microsoft Excel software). Each point on these scatter plots represented data from one drug for which both Phase II and preclinical activity values had been calculated from literature sources, as described above.

After descriptive evaluation of the data, Spearman rank correlation coefficients were obtained using the SAS software, UNIX version 6.12. A significance test of every correlation coefficient was performed, and the corresponding *P*s were calculated. Spearman rank (nonparametric) correlation coefficients were used because the distributions of the *x* (preclinical activity) and *y* (clinical activity) variables were not normal (29).

When multiple comparisons are made within a group of data such as in this work, there is increased possibility that some correlations will come up as statistically significant solely because of chance (false positives). To avoid this, multiple comparison correction methods (*e.g.*, Bonferroni approach) are often used to adjust the significance level to a lower *P* than conventionally used. However, relying on corrected probabilities increases the possibility that meaningful correlations will be missed (false negatives), making the nature of the scientific work key to the decision to use multiple comparison adjustment methods or not. Because this was an exploratory study, we were willing to accept a higher probability of false positives to ensure that potentially meaningful associations would not be discarded. We therefore did not correct for multiple comparisons and chose a level of significance of 0.05.

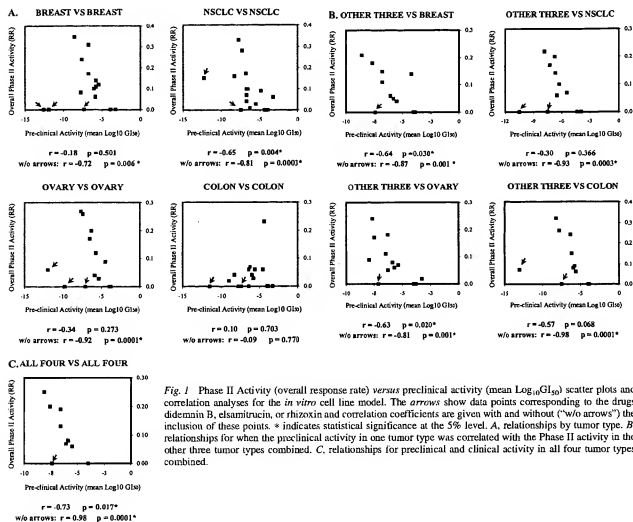


Fig. 1 Phase II Activity (overall response rate) versus preclinical activity (mean Log₁₀GI₅₀) scatter plots and correlation analyses for the *in vitro* cell line model. The arrows show data points corresponding to the drugs didemnin B, elasmuricin, or rhizoxin and correlation coefficients are given with and without ("w/o arrows") the inclusion of these points. * indicates statistical significance at the 5% level. A, relationships by tumor type. B, relationships for when the preclinical activity in one tumor type was correlated with the Phase II activity in the other three tumor types combined. C, relationships for preclinical and clinical activity in all four tumor types combined.

RESULTS

The Medline and CancerLit databases were searched for cancer drugs (excluding agents with novel targets such as signal transduction or angiogenesis modulators) that had undergone single agent Phase I clinical trial testing either in 1991 or 1992. This search led to 97 drug names. After excluding drugs that were eliminated from additional clinical testing for practical reasons (for example difficulties with the drug formulation), drugs that were specifically developed for a certain type of cancer (as for example hormone-regulating compounds for breast cancer) and drugs that were still the subject of published Phase I studies in 1991 and 1992 despite already being licensed for human use before 1985, a list of 31 agents was obtained (Table 1). After applying the restrictions and criteria mentioned under "Materials and Methods," we extracted from the literature preclinical and Phase II activity information for those agents on four common cancer types, breast, NSCLC, ovary, and colon. Overall, 100 preclinical and 307 Phase II clinical literature references were used spanning the period between 1985 and 2000.

No preclinical data were found for 5 of the 31 drugs researched. Of the 26 drugs remaining, availability of preclinical and Phase II data varied, depending on which preclinical and clinical tumor(s) had been tested and published in each case. Thus, each of the relationships examined had a different number of data points as different subsets of drugs were included. The most data points for any relationship were 17. For six relationships, five or fewer data points were available (relationships with fewer than five data points were not included in the results presented below).

In Vitro Cell Line Model. Fig. 1 shows the Phase II activity versus preclinical activity scatter plots and correlation analysis for the *in vitro* cell line model when the mean Log₁₀GI₅₀ was used as the measure of preclinical activity. Because the lower the mean Log₁₀GI₅₀, the higher the potency of a drug, a negative correlation between mean Log₁₀GI₅₀ and Phase II overall RR was expected if the model had a good clinical predictive value. Significant negative correlations were found for NSCLC (Fig. 1A), for breast or ovarian cell lines versus overall Phase II activity in the other three tumor types

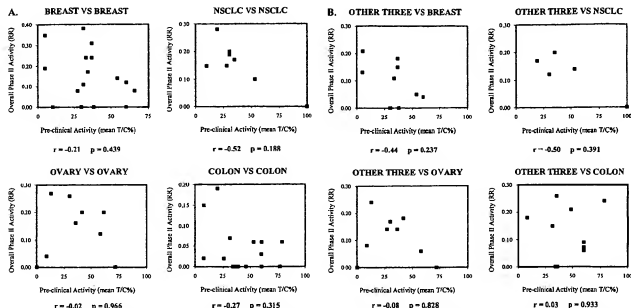


Fig. 2. Phase II activity (overall response rate) versus preclinical activity (mean T/C%) scatter plots and correlation analyses for the human xenograft model. A, relationships by tumor type. B, relationships when the preclinical activity in one tumor type was correlated with the Phase II activity in the other three tumor types combined.

(Fig. 1B), and for preclinical activity versus Phase II activity in all four tumor types (Fig. 1C).

Although the trends observed with the activity fraction were similar to ones seen for the mean $\text{Log}_{10}\text{GI}_{50}$ measure, no correlations were statistically significant in this case (data not shown).

Human Xenograft Model. A negative correlation between Phase II RRs and mean T/C% values was expected to be indicative of a good clinical predictive value for the human xenograft model. As shown in Fig. 2, no significant correlations between preclinical and clinical activity were observed for this model in our analysis.

For some of the drugs, preclinical activity calculations were based on multiple human xenografts of the same tumor type (i.e., panels) while for others on only a single xenograft. The relationships in Fig. 2 were reanalyzed, including only the drugs for which preclinical information on more than one human xenograft was available (Fig. 3). The results did not change for breast or colon tumors (compare Fig. 3A with Fig. 2A). However, the relationship for NSCLC became statistically significant and a highly significant correlation was seen for ovarian cancer (Fig. 3A). A near significant correlation was obtained when ovarian human xenograft panels were used to predict clinical activity in the other three tumor types combined (Fig. 3B).

Murine Allografts. No significant correlations between preclinical and clinical activity were observed for any of the relationships examined in this study for the murine allograft model (data not shown).

Additional Analyses. The scatter plots in Fig. 1 revealed an interesting observation: in every relationship except for colon

cancer under the disease oriented approach, an obvious trend toward a negative correlation was evident except for one to three outlier data points (Fig. 1, arrows). Interestingly, in all cases, these outlier data points corresponded to the same three drugs, namely elasmittucin, didemnin B, and rhizoxin.

In an attempt to provide a possible explanation for this observation, we considered the mechanism of action of all drugs that were included in the correlations in Fig. 1. From a total of 18 drugs (Table 2), 5, namely, elasmittucin, didemnin B, rhizoxin, flavone acetic acid, and fosquidone, were distinct in that they seemed to act through mostly unknown pathways that were not the typical DNA-based mechanisms of action of cytotoxic cancer agents. Thus, although flavone acetic acid and fosquidone fitted the rest of the data, there seemed to be a plausible mechanistic basis for the outlier behavior of the data points for elasmittucin, didemnin B, and rhizoxin. In fact, exclusion of these three drugs led to highly significant correlations in all cases except for the same tumor relationship in colon cancer (Fig. 1, correlation coefficients and P s for "w/o arrows"). It should be noted that none of the relationships examined for the human xenograft models (Figs. 2 and 3) included elasmittucin, didemnin B, or rhizoxin as data points.

Because of the intriguing results obtained with the human NSCLC and ovarian xenograft panels in Fig. 3A, a more detailed examination of these panels was pertained. As seen in Figs. 4A and 5A, the 6 ovarian and 7 NSCLC xenograft panels differed both in the numbers (minimum of 6 and maximum of 13 for ovary and minimum of 2 and maximum of 8 for NSCLC) and the identity of the xenografts that they contained. Analysis by grade/histology was hindered by lack of complete information on all xenografts. However, some patterns appeared distinguish-

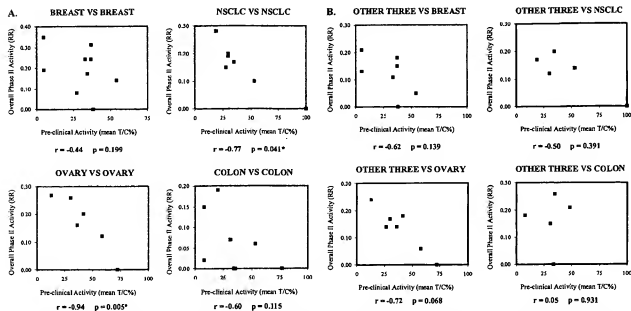


Fig. 3 Phase II activity (overall response rate) versus preclinical activity (mean T/C%) scatter plots and correlation analyses for the human xenograft model. Only data points for which two or more human xenografts were used to generate the preclinical activity values are shown. * indicates statistical significance at the 5% level. A, relationships by tumor type. B, relationships for when the preclinical activity in one tumor type was correlated with the Phase II activity in the other three tumor types combined.

Table 2 Mechanisms of action of drugs used in clinical vs. pre-clinical correlations for the *in vitro* cell line model (Fig. 1)
Atypical cytotoxics are shown in bold.

Drug	Mechanism of action
Amonafide	DNA intercalator
CI-921	Acts on topoisomerase II
Didemnin B	Not understood. Believed to act on protein synthesis
Elsamitricin	Not understood. It has been observed to inhibit topoisomerase I and II in <i>in vitro</i> experiments (relevance to <i>in vivo</i> uncertain). In cells in culture it has been observed to cause a cytostatic effect.
Epirubicin	Attaches to DNA at G bases
Fazarabine	Probably inhibits DNA synthesis by incorporation into DNA.
Flavone acetic acid	Has antiproliferative action in mice (probably not applicable to humans). Also believed to induce cell cycle arrest by generating reactive oxygen species that act on DNA.
Monogall	Causes cleavage of double-stranded DNA by inhibiting topoisomerase II
Piritrexim	Inhibits dihydrofolate reductase
Rhizoxin	Not fully understood. May interact with tubulin (different binding site than taxoids) and lead to cell cycle arrest. Also observed to act as an angiogenesis inhibitor.
Taxol	Microtubule destabilizing agent that causes apoptosis
Taxotere	Microtubule destabilizing agent that causes apoptosis
Teniposide	DNA synthesis inhibition by stabilization of cleavable DNA complexes
Topotecan	Topoisomerase I inhibitor
Trimetrexate	Antifolate
Fosquidone	Unknown. Not a DNA binder or a topoisomerase inhibitor
Tomudex	Thymidylate synthase inhibitor
Tiazofurin	Inhibits 5'-phosphodehydrogenase, the rate-limiting enzyme for guanine ribonucleotide synthesis

able. All ovarian panels contained 10–20% undifferentiated tumors and also included both poorly differentiated and moderately differentiated subtypes (Fig. 4B). For NSCLC, all panels included adenocarcinoma xenografts with a frequency of >30% (Fig. 5B). These observations suggested that the frequency of histological/grade subtypes within a xenograft panel may be an

important determinant of clinical predictivity rather than the number or the nature of the xenografts.

In an attempt to explore this hypothesis and to further examine the validity of the results obtained for ovarian cancer and NSCLC in Fig. 3A, the literature was reviewed for additional data. Six more agents with known overall Phase II RRs in

A.

NAME	HISTOLOGY / GRADE	DATA POINTS (DRUGS)					
		EPHIRUBICIN	POSQUIDONE	GEMCITABINE	MENOGARIL	TAXOTERE	PACLITAXEL
MRJ-4.207	undifferentiated	+	+		+	+	
A2780	undifferentiated	+		+	+		+
Ov.Hc	mod. diff., mucinous	+	+		+		
Ov.Me	carcinomatous	+	+	+			
Ov.RC	mod. diff., serous	+	+	+			
Fma	poorly diff., mucinous	+	+		+	+	
Ov.Pe	mod. diff., mucinous	+	+	+		+	
Fco	clear cell sarcoma	+	+		+		
T17	cystadenocarcinoma	+					
T385	adenocarcinoma	+					
OvGR	mod. diff., mucinous		+				
Fko	mod. diff., serous		+	+		+	
Ov.G1	poorly diff., serous		+			+	
OVCAR-3	adenocarcinoma			+		+	+
A121a	?					+	+
HOC18	poorly diff., serous					+	+
HOC22	undifferentiated					+	+
A2780/DDP	undifferentiated					+	+
A2780/DDX	adenocarcinoma					+	+
SKOV-3	cystadenocarcinoma					+	+
1°ovary 1	dediff. serous adeno.					+	+
1°ovary 2	moderately diff.					+	+
1GROV 1	poorly diff. adeno.					+	+
OVCAR-8	adenocarcinoma					+	+
OVCAR-5	poorly diff., serous					+	
Ovch	poorly diff., serous					+	
HOC22-S	poorly diff., serous					+	
TOTAL NO.		10	10	6	8	10	13

B.

HISTOLOGY/GRADE FREQUENCIES IN HUMAN OVARIAN XENOGRAFT PANELS							
HISTOLOGY / GRADE	EPHIRUBICIN NO. (%)	POSQUIDONE NO. (%)	GEMCITABINE NO. (%)	MENOGARIL NO. (%)	TAXOTERE NO. (%)	PACLITAXEL NO. (%)	
undifferentiated	2 (20)	1 (10)	1 (17)	2 (25)	1 (10)	3 (23)	
mod. diff., mucinous	2 (20)	3 (30)	2 (33)	2 (25)	1 (10)	0 (0)	
mod. diff., serous	1 (10)	2 (20)	2 (33)	1 (12.5)	1 (10)	0 (0)	
poorly diff., mucinous	1 (10)	1 (10)	0 (0)	1 (12.5)	1 (10)	0 (0)	
poorly diff., serous	0 (0)	1 (10)	0 (0)	0 (0)	4 (40)	2 (15)	
unspecified	4 (40)	2 (20)	1 (17)	2 (25)	2 (20)	8 (62)	
TOTAL	10 (100)	10 (100)	6 (100)	8 (100)	10 (100)	13 (100)	

Fig. 4 Human ovarian xenograft panels for the six data points (drugs) used in the "Ovary versus Ovary" relationship in Fig. 3A. A, names and histology/grade (? = unknown, mod. diff. = moderately differentiated, poorly diff. = poorly differentiated, dediff. = dedifferentiated, adeno. = adenocarcinoma) of all of the xenografts tested. Inclusion of a particular xenograft in one of the panels is shown by a "+" sign in the corresponding row and under the appropriate drug column. B, histology/grade subtypes in the human ovarian xenograft panels by number and percentage.

previously treated patients with ovarian cancer were found. Five and one of these compounds had been tested in a panel of 15 and 6 human ovarian xenografts, respectively (26, 30), which fitted the histology/grade patterns identified in Fig. 4B. Fig. 6A lists the names and Phase II RRs (31–56) of these additional drugs together with the six compounds that were included in the analysis in Fig. 3A. Fig. 6, A and B, also shows mean T/C% values scatter plots and statistical analyses for two cases: first, for when all of the available xenograft information was used, and second, for when mean T/C% calculations were based, where possible, on the arithmetically smallest panel, namely the one used for gemcitabine in Fig. 4. Highly significant correlations were obtained in both cases (Fig. 6B).

For NSCLC information on two additional agents was found: amscrine [mean T/C% of 62 (26) and Phase II RR equal to 0.06 (31)] and doxorubicin [mean T/C% of 47 (26) and Phase II RR equal to 0.12 (32)]. Both had been tested in NSCLC human xenograft panels that included all three histological subtypes and had adenocarcinoma contents of 29 and 33%,

respectively. As for ovarian cancer, those two additional data points (Fig. 5C, arrows) enhanced the statistical significance of the relationship observed in Fig. 3A.

DISCUSSION

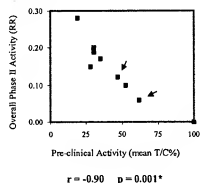
A literature-based, retrospective study was conducted to examine the clinical predictive value of three widely used preclinical cancer models, namely, the *in vitro* human tumor cell line, the human xenograft, and the murine allograft models. Four solid tumor types were selected, breast, NSCLC, ovary and colon, and data on a set of 31 anticancer agents (excluding agents with novel targets such as signal transduction or angiogenesis modulators) were collected. Preclinical activity in each model was correlated with RRs in Phase II clinical trials by tumor type (disease-oriented approach) in the case when one preclinical tumor type was used as a predictor of overall clinical activity in the other three tumor types combined (compound-oriented approach) and for all four tumor types together.

Fig. 5 Human NSCLC xenograft panels for the seven data points (drugs) used in the NSCLC versus NSCLC relationship in Fig. 3A. A, drug names (EPI = epirubicin, FAZ = fazarabine, GEM = gemcitabine, IRINO = irinotecan, PACLIT = paclitaxel, TOPO = topotecan, VINRLB = vinorelbine) and histological subtypes (? = unknown) of all of the xenografts tested. Inclusion of a particular xenograft in one of the panels is shown by a "+" sign in the corresponding row and under the appropriate drug column. B, histological subtypes in the human NSCLC xenograft panels by number and percentage. C, scatter plot and correlation analysis for the same tumor clinical versus preclinical activity relationship in NSCLC, including the seven drugs in Fig. 6A as well as two additional agents, doxorubicin and amiracrine (data points shown with arrows), with known NSCLC Phase II and human xenograft activities.

XEN. NAME	XENOGRAFT HISTOLOGY	DRUGS						
		EPI	FAZ	GEM	IRINO	PACLIT	TOPO	VINRLB
T222	squamous cell	+						
T291	adenocarcinoma	+						
UCLA-P3	adenocarcinoma		+					
ACCOLU-78	squamous cell		+					
NCI-H460	large cell			+	+	+	+	+
A549	adenocarcinoma			+	+	+	+	+
CaLo-6	adenocarcinoma			+				
H-74	?			+				
LC-576	?			+				
QG-56	squamous cell				+			+
NCI-H23	adenocarcinoma				+	+		
NCI-H226	squamous cell				+		+	
MY-522	adenocarcinoma					+		
CaLo-3	adenocarcinoma					+		
1° NSCLC	adenocarcinoma					+		
L2987	adenocarcinoma					+		
L-57	adenocarcinoma							+
LC-06	large cell							+
LU-65	large cell							+
PC-12	adenocarcinoma							+
LU-99	large cell							+
TOTAL NO.		2	2	5	5	8	3	8

HISTOLOGY FREQUENCY IN HUMAN NSCLC XENOGRAFT PANELS								
HISTOLOGY	EPI NO. (%)	FAZ NO. (%)	GEM NO. (%)	IRINO NO. (%)	PACLIT NO. (%)	TOPO NO. (%)	VINORLB NO. (%)	
adenocarcinoma	1 (50)	1 (50)	2 (40)	2 (40)	6 (75)	1 (33.3)	3 (37.5)	
large cell	0 (0)	0 (0)	1 (20)	1 (20)	1 (12.5)	1 (33.3)	4 (50)	
squamous cell	1 (50)	1 (50)	0 (0)	2 (40)	1 (12.5)	1 (33.3)	1 (12.5)	
unknown			2 (40)					
TOTAL	2 (100)	2 (100)	5 (100)	5 (100)	8 (100)	3 (100)	8 (100)	

C. NSCLC VS NSCLC (ADDITIONAL DATA)



Colon cancer was the only site for which a disproportional amount of clinically active versus inactive agents were identified: only 3 drugs with Phase II RRs > 0.15 and 8 with ≤ 0.10 (Figs. 1-3). However, this was likely a reflection of the lack of clinically effective drugs for this tumor type rather than the result of selection and publication bias.

When the mean $\text{Log}_{10}\text{GI}_{50}$ measure of preclinical activity was used, the *in vitro* cell line model was found to be predictive

of Phase II clinical performance for NSCLC under the disease-oriented approach in breast and ovarian cancers under the compound-oriented approach and in the case of all four tumor types together. Highly significant correlations were observed in all cases, except colon cancer, when three consistent outlier data points corresponding to the mechanistically nontypical cytotoxic agents didenmin B, elasmirucina, and rhizoxin were excluded in exploratory analysis. Thus, the *in vitro* cell line model

A.

DRUG	PHASE II RESPONSE RATE	HUMAN OVARIAN XENOGRAFT MEAN TC%	
		ALL TESTED	GEMCITABINE PANEL
STUDY DRUGS	EPIDORUBICIN	42	-
	FOXQUIDONE	72	-
	GEMCITABINE	36	36
	MENOGARIL	58	-
	PACLITAXEL	30	-
	TAXOTERE	13	-
ADDITIONAL DRUGS	DOXORUBICIN	47 ^{ns}	47 ^{ns}
	AMISACRINE	75 ^{ns}	-
	CISPLATIN	41 ^{ns}	46 ^{ns}
	HEXAMETHYL- MELAMINE	28 ^{ns}	31 ^{ns}
	METHOTREXATE	76 ^{ns}	54 ^{ns}
	5-FU	71 ^{ns}	71 ^{ns}

B.

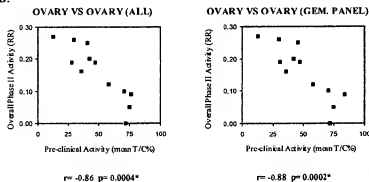


Fig 6 A, preclinical and Phase II clinical activity data for ovarian cancer, including the six drugs in Fig. 3A ("Study Drugs") as well as an additional six drugs ("Additional Drugs") with known ovarian Phase II and human xenograft activities. Literature references are shown in superscript font. B, scatter plots and correlation analysis for the same tumor clinical versus preclinical activity relationship in ovarian cancer based on the data in Fig. 5A. Analysis was done for (a) when all of the xenografts were included in preclinical activity calculations ("All") and (b) when only the six xenografts in the gemcitabine panel were used for preclinical activity calculations, where possible ("Gem. Panel"). Stars indicate statistical significance at the 5% level.

might be predictive in the case of typical cytotoxic cancer agents but might fail to provide reliable information for at least some of the noncytotoxic cancer drugs. Additional studies are needed to explore this observation.

The fact that drug potency (mean $\text{Log}_{10}\text{GI}_{50}$), a pharmacological measure, was found to be predictive of Phase II performance was somewhat surprising but has been noted previously: a recent study by Johnson *et al.* (18) demonstrated a highly significant correlation between potency in the NCI human tumor cell line screen and activity in the hollow fiber assay. Pharmacological considerations (pharmacological differences between the species) might provide a possible explanation why some anticancer agents appear effective in *in vivo* mouse models but fail to show efficacy in Phase II trials. Experience with some agents (57) has shown that the maximum-tolerated dose in mouse can be higher than in humans, presumably because of an intrinsic ability of mouse cells to tolerate higher drug doses and/or more efficient elimination in the mouse.

In contrast to the *in vitro* cell line, our results suggest that the murine allograft model, as used in this analysis, is not predictive of clinical Phase II performance. This is in agreement with the conclusions from a large body of information originating from the NCI screening programs in use from 1975 to 1990 (5-8, 10-12).

The human xenograft model showed good tumor-specific predictive value for NSCLC and ovarian cancers when panels of xenografts were used. However, it failed to adequately predict clinical performance both in the disease and compound-oriented settings for breast and colon tumors. The results with breast cancer were in agreement with a recent study (18) but were contradictory to the work reported by Bailey *et al.* (20), Inoue *et al.* (21), and Mattern *et al.* (24). However, given that the latter studies did not use formal statistical methods, our conclusions may be more robust. The results for ovarian cancer were in agreement with studies by Taetle *et al.* (23) and Mattern *et al.* (24) but contradicted the conclusions of the recent NCI United States study by Johnson *et al.* (18). Our results for NSCLC were consistent with the observations from all previous studies that examined same tumor correlations in this cancer type (18, 24).

For NSCLC and ovarian cancer patients, a panel of xenografts was more predictive than single xenografts confirming preliminary observations by Bellet *et al.* (19).

In an effort to identify the properties that may render an ovarian or NSCLC human xenograft panel predictive of Phase II drug performance, common characteristics were sought. There was no similarity in number and only limited overlap in identity of xenografts between same tumor type panels. However, certain patterns in histology/grade content were found. These ob-

servations suggest that the relative histology/grade content rather than the number or identity of xenografts within a panel may be the important determinant of clinical predictivity. To our knowledge, no other study has attempted to identify ovarian or NSCLC human xenograft panel features that might lead to accurate predictions of a drug's Phase II performance.

This is the only study that has examined the clinical predictive value of three preclinical cancer models together and thus allows for direct comparisons between them. The results suggest that the human xenograft model is more predictive than its murine allograft counterpart and that the *in vitro* cell line model is of, at least, equivalent usefulness to the human xenograft model.

The NCI work with cancer drug screening programs from 1955 to 1990 (Refs. 5-8, 10-12; leukemia-based preclinical, compound-oriented screens preferentially yielding compounds active against hematological malignancies) in combination with our work and recent conclusions by Johnson *et al.* (Ref. 18; statistically significant results under the compound-oriented approach for some solid tumors) suggest that the compound-oriented strategy may be successful when used only within solid tumors or only within hematological malignancies but not when the two disease groups are considered together.

In general, our results suggest that the *in vitro* human tumor cell line and the human xenograft models might have good clinical predictive value in some solid tumors (such as ovary and NSCLC) under both the disease and compound-oriented strategies, as long as an appropriate panel of tumors is used in preclinical testing.

In conclusion, given the results in this study and those of others (6, 7, 10-12), continued use of the murine allograft model in drug development may not be justified. The work presented here argues for emphasis to be placed on *in vitro* cell lines (in the context of the NCI Human Tumor Cell Line Screen) and appropriate panels of the human xenograft model.

Recent years have seen an explosion in the molecular understanding of cancer, which has led to the development of not only more effective cytotoxic cancer drugs but of potentially cytostatic or antimetastatic agents as well. The future preclinical and clinical development of traditional cytotoxic compounds will likely follow similar procedures with those practiced today, and in that sense, the present findings could contribute to the more efficient discovery of such agents. However, the existing cancer models and parameters of activity in both the preclinical and clinical settings may have to be redesigned to fit the mode of action of the novel cytostatic, antimetastatic, antiangiogenesis, or immune response-modulating agents (58). In the preclinical cancer model front, the case is being made for the use of the orthotopic mouse xenograft and transgenic models (59-61) because those are thought to more accurately simulate human disease, especially in terms of growth characteristics and metastatic behavior. New end points of preclinical activity are contemplated such as the demonstration that a new molecule truly hits the intended molecular target (58). In Phase II clinical trials, there is a growing effort toward validating new surrogate end points of drug efficacy (58). The next decade will probably answer many of the questions regarding the effectiveness of these novel agents and will likely define a new role for tradi-

tional cytotoxic therapies, but it will also bring new challenges in terms of preclinical predictors of activity.

REFERENCES

1. Curt, G. A. The use of animal models in cancer drug discovery and development. *Stem Cells*, 12: 23-29, 1994.
2. Goldin, A., Serpick, A. A., and Mantel, N. Experimental screening procedures and clinical predictability value. *Cancer Chemother. Rep.*, 50: 173-218, 1966.
3. Venditti, J. M. Drug evaluation branch program: report to screening contractors. *Cancer Chemother. Rep.*, 5: 1-4, 1975.
4. Venditti, J. M. Preclinical drug development: rationale and methods. *Semin. Oncol.*, 8: 349-353, 1981.
5. Venditti, J. M., Wesley, R. A., and Plowman, J. Current NCI preclinical antitumor screening *in vivo*: results of tumor panel screening, 1976-1982 and future directions. *Adv. Pharmacol. Chemother.*, 20: 1-19, 1984.
6. Venditti, J. M. The National Cancer Institute antitumor drug discovery program, current and future perspectives: a commentary. *Cancer Treat. Rep.*, 67: 767-772, 1983.
7. Staquet, M. J., Byar, D. P., Green, S. B., and Rozenzweig, M. Clinical predictivity of transplantable tumor systems in the selection of new drugs for solid tumors: rationale for a three-stage strategy. *Cancer Treat. Rep.*, 67: 753-765, 1983.
8. Goldin, A., Venditti, J. M., MacDonald, J. S., Muggia, F. M., Henney, J. E., and De Vita V. T. Current results of the screening program at the division of cancer treatment, National Cancer Institute. *Eur. J. Cancer*, 17: 129-142, 1981.
9. Marsoni, S., and Wittes, R. Clinical development of anticancer agents: A National Cancer Institute perspective. *Cancer Treat. Rep.*, 68: 77-85, 1983.
10. De Vita V. T., and Schein, P. S. The use of drugs in combination for the treatment of cancer. *N. Engl. J. Med.*, 288: 998-1006, 1973.
11. Zubrod, C. G. Chemical control of cancer. *Proc. Natl. Acad. Sci. USA*, 69: 1042-1047, 1972.
12. Marsoni, S., Hoth, D., Simon, R., Leyland-Jones, B., De Rosa, M., and Wittes, R. E. Clinical drug development: an analysis of Phase II trials, 1970-1985. *Cancer Treat. Rep.*, 71: 71-80, 1987.
13. Shoemaker, R. H., Monks, A., Alley, M. C., Scudiero, D. A., Fine, D. L., McLemore, T. L., Abbott, B. J., Paull, K. D., Mayo, J. G., and Boyd, M. R. Development of human tumor cell line panels for use in disease-oriented drug screening. *Prog. Clin. Biol. Res.*, 276: 265-286, 1988.
14. Skehan, P., Storey, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J. T., Bokesch, H., Kenney, S., and Boyd, M. R. New colorimetric cytotoxicity assay for anticancer drug screening. *J. Natl. Cancer Inst.* (Bethesda), 82: 1107-1112, 1990.
15. Rubinstein, L. V., Shoemaker, R. H., Paull, K. D., Simon, R. M., Tosini, S., Skehan, P., Scudiero, D., Monks, A., and Boyd, M. R. Comparison of *in vitro* anticancer drug screening data generated with a tetrazolium assay versus a protein assay against a diverse panel of human tumor cell lines. *J. Natl. Cancer Inst.* (Bethesda), 82: 1113-1118, 1990.
16. Alley, M. C., Scudiero, D., Monks, A., Hursey, M. L., Czerwinski, M. J., Fine, D. L., Abbott, B. J., Mayo, J. G., Shoemaker, R. H., and Boyd, M. R. Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Res.*, 48: 589-601, 1988.
17. Monks, A., Scudiero, D., Skehan, P., Shoemaker, R. H., Paull, K. D., Vistica, D., Hose, C., Langley, J., Cronise, P., Vaigro-Wolf, A., Gray-Goodrich, M., Campbell, H., Mayo, J., and Boyd, M. R. Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. *J. Natl. Cancer Inst.* (Bethesda), 83: 757-766, 1990.
18. Johnson, J. I., Decker, S., Zaharevitz, D., Rubinstein, L. V., Venditti, J. M., Schepartz, S., Kalyandurg, S., Christin, M., Arbuck, S., Hollingshead, M., and Sausville, E. A. Relationships between drug

- activity in NCI preclinical *in vitro* and *in vivo* models and early clinical trials. *Br. J. Cancer*, 84: 1424-1431, 2001.
19. Bellet, R. E., Danna, V., Mastrangelo, M. J., and Berd, D. Evaluation of a "nude" mouse-human tumor panel as a predictive secondary screen for cancer chemotherapeutic agents. *J. Natl. Cancer Inst. (Bethesda)*, 63: 1185-1187, 1979.
20. Bailey, M. J., Gazet, J.-C., Smith, I. E., and Steel, G. G. Chemotherapy of human breast-carcinoma xenografts. *Br. J. Cancer*, 42: 530-536, 1980.
21. Inoue, K., Fujimoto, S., and Ogawa, M. Antitumor efficacy of seventeen anticancer drugs in human breast cancer xenograft (MX-1) transplanted in nude mice. *Cancer Chemother. Pharmacol.*, 10: 182-186, 1983.
22. Steel, G. G., Courtenay, V. D., and Peckham, M. J. The response to chemotherapy of a variety of human tumor xenografts. *Br. J. Cancer*, 47: 1-13, 1983.
23. Taetle, R., Rosen, F., Abramson, I., Venditti, J., and Howell, S. Use of nude mouse xenografts as preclinical drug screens: *in vivo* activity of established chemotherapeutic agents against melanoma and ovarian carcinoma xenografts. *Cancer Treat. Rep.*, 71: 297-304, 1987.
24. Mattern, J., Bak, M., Hahn, E. W., and Volm, M. Human tumor xenografts as models for drug testing. *Cancer Metastasis Rev.*, 7: 263-284, 1988.
25. Boven, E., Winograd, B., Fodstad, O., Lobbezoo, M. W., and Pinedo, H. M. Preclinical Phase II studies in human tumor lines: a European multicenter study. *Eur. J. Cancer*, 24: 567-573, 1988.
26. Boven, E., Winograd, B., Berger, D. P., Dumant, M. P., Brakhuus, B. J. M., Fodstad, O., Langdon, S., and Fiebig, H. H. Phase II preclinical drug screening in human tumor xenografts: a first European multicenter collaborative study. *Cancer Res.*, 52: 5940-5947, 1992.
27. Langdon, S., Hendriks, H. R., Brakhuus, B. J. M., Pratesi, G., Berger, D. P., Fodstad, O., Fiebig, H., and Boven, E. Preclinical Phase II studies in human tumor xenografts: a European multicenter follow-up study. *Ann. Oncol.*, 5: 415-422, 1994.
28. Paull, K. D., Shoemaker, R. H., Hodges, L., Monks, A., Scudiero, D., Rubinstein, L. V., Plowman, J., and Boyd, M. R. Display and analysis of patterns of differential activities of drugs against human tumor cell lines: development of mean graph and COMPARE algorithm. *J. Natl. Cancer Inst. (Bethesda)*, 81: 1088-1092, 1989.
29. Rosner, B. Non-parametric Methods. In: *Fundamentals of Biostatistics*, 4th ed., p. 575. Belmont, CA: Duxbury Press, Wadsworth Publishing Company, 1994.
30. Kolfschoten, G. M., Pinedo, H. M., Scheffer, P. G., Schluper, H. M. M., Erkelens, C. A. M., and Boven, E. Development of a panel of 15 human ovarian cancer xenografts for drug screening and determination of the role of the glutathione detoxification system. *Gynecol. Oncol.*, 76: 362-368, 2000.
31. Louie, A. C., and Isell, B. F. Amisarin (AMSA): a clinical review. *J. Clin. Oncol.*, 3: 562-592, 1985.
32. Minna, J. D., Pass, H., Glatstein, E., and Inde, D. Cancer of the lung. In: V. T. DeVita, S. Hellman, and S. A. Rosenberg (eds.), *Cancer: Principles and Practice of Oncology*, pp. 660 (Table 22-48). Philadelphia: J. B. Lippincott, 1989.
33. Gordon, A. N., Grana, C. O., Rose, P. G., Hainsworth, J. Lopez, A., Weissman, C., Rosales, R., and Sharpington, T. Phase II study of liposomal doxorubicin in platinum- and paclitaxel-refractory epithelial ovarian cancer. *Clin. Oncol.*, 18: 3093-3100, 2000.
34. Israel, V. P., Garcia, A. A., Roman, L., Muderspach, L., Burnett, A., Jeffers, S., and Muggia, F. M. Phase II study of liposomal doxorubicin in advanced gynecologic cancers. *Gynecol. Oncol.*, 78: 143-147, 2000.
35. Muggia, F. M., Hainsworth, J. D., Jeffers, S., Miller, P., Groshen, S., Tan, M., Roman, L., Uziely, B., Muderspach, L., Garcia, A., Burnett, A., Greco, F. A., Morrow, C. P., Paradiso, L. J., and Liang, L. J. Phase II study of liposomal doxorubicin in refractory ovarian cancer: antitumor activity and toxicity modification by liposomal encapsulation. *J. Clin. Oncol.*, 15: 987-993, 1997.
36. Blum, R. H., and Carter, S. K. Adriamycin: a new anticancer drug with significant clinical activity. *Ann. Intern. Med.*, 80: 249-259, 1974.
37. Thigpen, J. T., Lagasse, L., Homesley, H., and Blessing, J. A. Cis-platinum in the treatment of advanced or recurrent adenocarcinoma of the ovary. A Phase II study of the Gynecologic Oncology Group. *Am. J. Clin. Oncol.*, 6: 431-435, 1983.
38. Willshaw, E., and Kroner, T. Phase II study of cis-dichlorodiamminoplatinum(II) (NSC-119875) in advanced adenocarcinoma of the ovary. *Cancer Treat. Rep.*, 60: 55-60, 1976.
39. Bonomi, P. D., Mladineo, J., Morrin, B., Wilbanks, G. Jr., and Slayton, R. E. Phase II trial of hexamethylmelamine in ovarian carcinoma resistant to alkylating agents. *Cancer Treat. Rep.*, 63: 137-138, 1979.
40. Johnson, B. L., Fisher, R. I., Bender, R. A., DeVita, V. T., Jr., Chabner, B. A., and Young, R. C. Hexamethylmelamine in alkylating agent-resistant ovarian carcinoma. *Cancer (Phila.)*, 42: 2157-2161, 1978.
41. Omura, G. A., Blessing, J. A., Morrow, C. P., Buchsbaum, H. J., and Homesley, H. D. Follow-up on a randomized trial of melphalan (M) vs. melphalan plus hexamethylmelamine (M+H) versus Adriamycin plus cyclophosphamide (A+C) in advanced ovarian carcinoma. *Proc. Am. Assoc. Cancer Res.*, 22: 470, 1981.
42. Bolis, G., D'Incalci, M., Belloni, C., and Mangioni, C. Hexamethylmelamine in ovarian cancer resistant to cyclophosphamide and Adriamycin. *Cancer Treat. Rep.*, 63: 1375-1377, 1979.
43. Manetta, A., MacNeill, C., Lyter, J. A., Schettler, B., Podczaski, E. S., Larson, J. E., and Schein, P. Hexamethylmelamine as a single second-line agent in ovarian cancer. *Gynecol. Oncol.*, 36: 93-96, 1990.
44. Rosen, G. F., Lurain, J. R., and Newton, M. Hexamethylmelamine in ovarian cancer after failure of cisplatin-based multiple-agent chemotherapy. *Gynecol. Oncol.*, 27: 173-179, 1987.
45. Parker, L. M., Griffiths, C. T., Yankee, R. A., Knapp, R. C., and Candellos, G. P. High-dose methotrexate with leucovorin rescue in ovarian cancer: a Phase II study. *Cancer Treat. Rep.*, 63: 275-279, 1979.
46. Barlow, J. J., and Piver, M. S. Methotrexate (NSC-740) with citrovorum factor (NSC-3590) rescue, alone and in combination with cyclophosphamide (NSC-26271), in ovarian cancer. *Cancer Treat. Rep.*, 60: 527-533, 1976.
47. Morgan, R. J., Jr., Speyer, J., Doroshow, J. H., Margolin, K., Raschko, J., Sorich, J., Akman, S., Leong, L., Soulo, G., and Vastly, S. Modulation of 5-fluorouracil with high-dose leucovorin calcium: activity in ovarian cancer and correlation with CA-125 levels. *Gynecol. Oncol.*, 58: 79-85, 1995.
48. Markman, M., Reichman, B., Hakes, T., Hoskins, W., Rubin, S., Jones, W., and Lewis, J. R. Intraperitoneal chemotherapy as treatment for ovarian carcinoma and gastrointestinal malignancies: the Memorial Sloan-Kettering Cancer Center experience. *Acta Med. Austriaca*, 16: 65-67, 1989.
49. Prefontaine, M., Donovan, J. T., Powell, J. L., and Buley, L. Treatment of refractory ovarian cancer with 5-fluorouracil and leucovorin. *Gynecol. Oncol.*, 61: 249-252, 1996.
50. Kamphuis, J. T., Huider, M. C., Ras, G. J., Verhagen, C. A., Katenam, I., Vreeswijk, J. H., and Burghouts, J. T. High-dose 5-fluorouracil and leucovorin as second-line chemotherapy in patients with platinum-resistant epithelial ovarian cancer. *Cancer Chemother. Pharmacol.*, 37: 190-192, 1995.
51. Burnett, A. F., Barter, J. F., Potkul, R. K., Jarvis, T., and Barnes, W. A. Ineffectiveness of continuous 5-fluorouracil as salvage therapy for ovarian cancer. *Am. J. Clin. Oncol.*, 17: 490-493, 1994.
52. Reed, E., Jacob, J., Ozols, R. F., Young, R. C., and Allegra, C. 5-Fluorouracil (5-FU) and leucovorin in platinum-refractory advanced stage ovarian carcinoma. *Gynecol. Oncol.*, 46: 326-329, 1992.
53. Ozols, R. F., Speyer, J. L., Jenkins, J., and Myers, C. E. Phase II trial of 5-FU administered ip to patients with refractory ovarian cancer. *Cancer Treat. Rep.*, 68: 1229-1232, 1984.

54. Long, H. J., III, Nelmark, R. A., Su, J. Q., Garneau, S. C., Levitt, R., Goldberg, R. M., Poon, M. A., and Kugler, J. W. Phase II evaluation of 5-fluorouracil and low-dose leucovorin in cisplatin-refractory advanced ovarian carcinoma. *Gynecol. Oncol.*, 54: 180-183, 1994.
55. Look, K. Y., Muss, H. B., Blessing, J. A., and Morris, M. A. Phase II trial of 5-fluorouracil and high-dose leucovorin in recurrent epithelial ovarian carcinoma. A Gynecology Oncology Group Study. *Am. J. Clin. Oncol.*, 18: 19-22, 1995.
56. De Gneff, A., van Hoef, M. E., Tjia, P., Heintz, A. P., and Neijt, J. P. Continuous infusion of 5-fluorouracil in ovarian cancer patients refractory to cisplatin and carboplatin. *Ann. Oncol.*, 2: 691-692, 1991.
57. Volpe, D. A., Tomaszewski, J. E., Parchment, R. E., Garg, A., Flora, K. P., Murphy, M. J., and Grieshaber, C. K. Myelotoxic effects of the bifunctional alkylating agent bizelesin in human, canine and murine myeloid progenitor cells. *Cancer Chemother. Pharmacol.*, 39: 143, 1996.
58. Gelmon, K. A., Eisenhauer, E. A., Harris, A. L., Ratain, M. J., and Workman, P. Anticancer agents targeting signaling molecules and cancer cell environment: challenges for drug development? *J. Natl. Cancer Inst.* (Bethesda), 91: 1281-1287, 1999.
59. Killian, J. J., Radinsky, R., and Fidler, I. J. Orthotopic models are necessary to predict therapy of transplantable tumors in mice. *Cancer Metastasis Rev.*, 17: 279-284, 1999.
60. Kerbel, R. S. What is the optimal rodent model for anti-tumor drug testing? *Cancer Metastasis Rev.*, 17: 301-304, 1999.
61. Rosenberg, M. P., and Bortner, D. Why transgenic and knockout animal models should be used (for drug efficacy studies in cancer). *Cancer Metastasis Rev.*, 17: 295-299, 1999.

Oncologic, Endocrine & Metabolic



<http://www.ashley-pub.com>

Review

- 1. Introduction
- 2. Tumour antigens
- 3. Monitoring immune response and heterogeneity
- 4. Assays of bulk cultures of peripheral lymphocytes
- 5. Functional analysis of TA-specific T-cells by gene expression
- 6. *In vivo* biologic measures of T-lymphocyte responses
- 7. Summary
- 8. Bibliography

T-cell-directed cancer vaccines: the melanoma model

Ena Wang, Giao Q Phan & Francesco M Marincola

Surgery Branch, National Cancer Institute, Bethesda, Maryland, USA

Significant advances in the understanding of the molecular basis for tumour/host interactions in humans have occurred in the last decade through studying patients with metastatic melanoma. This disease is characterised by its tendency to be modulated by immunologic factors. Furthermore, immunologic manipulation of the host with various systemic agents, in particular IL-2, frequently affects this natural phenomenon and can lead to complete rejection of cancer. By studying the cellular immunology occurring in patients undergoing immunotherapy, several tumour antigens (TA) and their epitopes recognised by human leukocyte antigen (HLA) class I-restricted cytotoxic T-lymphocytes (CTL) have been identified. Most of these TA are non-mutated molecules expressed by the majority of melanoma *in vivo* and most melanoma cell lines. In addition, unique minimal epitopic sequences play an immunodominant role in the context of specific HLA class I alleles. Since melanoma lesions from different patients often share expression of the same TA, and a minimal peptide sequence from a TA can cause immunologic changes in multiple patients, interest has grown in the development of TA-specific vaccines suitable for broad patient populations. Repeated *in vitro* stimulation of peripheral blood mononuclear cells (PBMC) with TA-derived epitopes can induce a high frequency of TA-reactive T-cells in melanoma patients. The same epitopes can also enhance TA-specific T-cell reactivity *in vivo* when administered subcutaneously in combination with Incomplete Freund's Adjuvant (IFA). Epitope-based vaccinations, however, have not shown strong clinical efficacy unless combined with IL-2 administration. Attempts to increase the efficacy of these vaccines have combined specialised antigen-presenting cells or the administration of whole TA through DNA- or RNA-based vaccines with the intention of increasing antigen presentation and processing. Save for scattered reports, however, the success of these approaches has been limited and T-cell-directed vaccination against cancer remains at a paradoxical standstill whereby anticancer immunisation can be induced but it is not sufficient, in most cases, to induce tumour regression. Using melanoma as the standard model for immunotherapy, we will review various methods of T-cell-directed vaccination, the monitoring and analysis of the resulting immune response, and several clinical trials in which cancer vaccines have successfully induced immunisation.

Keywords: immune monitoring, immunotherapy, melanoma, neoplasm, T-lymphocyte, vaccines

Exp. Opin. Biol. Ther. (2001) 1(2):277-290

1. Introduction

The immune response against pathogens utilises both a humoral and a cellular arm. While the former is directed toward extracellular pathogens, the latter is directed toward proteins produced by infectious agents replicating within permissive host cells. Intracellular proteins produced by infectious agents are enzymatically degraded into short peptides (9–11 amino acids in length) and presented on the surface of infected host cells in association with Major Histocompatibility Complex (MHC) class I molecules. This MHC complex is required for triggering T-cells through direct interaction with the T-cell receptor (TCR). Most tumour antigens identified so far are intracellular molecules. Thus, the cellular immune response is likely the prevalent immunologic defence against cancer [1] since antibodies cannot penetrate the intracellular compartment.

Using IL-2, stable human T-cell lines specifically recognising autologous tumour cells could be expanded *in vitro* from excised tumour specimens [2]. Due to the capacity of IL-2 in enhancing T-cell activation, proliferation and antitumour activity *in vitro*, IL-2 was administered to patients with advanced cancer [3], in particular metastatic melanoma and renal cancer for which no effective alternative therapy is available [4]. Administration of high-dose iv. IL-2 resulted in 7% complete and 10% partial tumour regressions in patients with metastatic melanoma [5]. IL-2 was then used to expand *in vitro* lymphokine-Activated Killer (LAK) cells from peripheral blood monocytes (PBMC) [6] and tumour-infiltrating lymphocytes (TIL) from tumour specimens for adoptive cell transfer [7]. While LAK cells did not prove useful in randomised clinical trials [6], the adoptive transfer of TIL suggested an additional clinical benefit over of IL-2 alone inducing a 34% objective response rate [5].

Due to these promising results and the ease with which tumour-specific TIL could be obtained from melanoma patients, melanoma has served as the prototype human model for studying tumour immunology. Molecular characterisation of TIL led to identification of TA [2,7,8]. Kawakami *et al.* had shown that TIL could kill melanoma cell lines from different patients provided they expressed at least one matched MHC (called Human Leukocyte Antigen or HLA in humans) class I molecule [9]. Based on this HLA 'restriction', TA were identified. HLA-restricted recognition of TA was formally proven by transduction of a

specific HLA allele into melanoma cells lacking the 'restricting element' that was associated with recognition of the TA by a particular TIL [9]. After establishment of a cDNA library from the melanoma cell line recognised by the TIL, genes from the library were transfected into non-melanoma cells expressing the correct restriction element. The transfected target cells were then tested for recognition by the same TIL and the cDNA clones that caused recognition were isolated and sequenced for identification of the TA [5,10].

2. Tumour antigens

Two major categories of TA were identified. The first category includes tumour differentiation antigens (TDA), such as MART-1/MelanA, gp100/Pmel17, tyrosinase, TRP-1 and TRP-2. TDA are expressed by both melanoma cells and normal melanocytes [11–14] but not other malignancies and normal tissues [15]. The second category involves tumour specific antigens (TSA), which are found in normal gametic cells in the testes and in cancers in addition to melanoma but are not expressed by normal melanocytes. TSA include the MAGE, BAGE and GAGE families and NY-ESO-1 [16]. NY-ESO-1 was found in approximately 30% of melanomas and breast tumours and occasionally identified in malignancies of the lung, liver, thyroid, ovary and prostate [16]. Therefore, ESO-1 is a promising candidate tumour antigen and is currently the focus of numerous clinical trials.

Due to the prevalence of HLA-A*0201 in the melanoma population (~50%), a large number of TA were identified in association with this allele [17,18]. Among them, MART-1 has received particular attention because of its 'immunodominance' in the context of HLA-A*0201 [19]. It was originally noted that 90% of TIL expanded from HLA-A*0201 patients recognise the MART-1:27–35 epitopic sequence [20]. This work was based on the analysis of archival TIL strains maintained in culture for several passages. More recent work by Kawakami *et al.* extended the analysis of specificity to a broader population of short-term HLA-A*0201-associated TIL cultures and suggested that the rate of MART-1 recognition is not as high as originally believed [21]. We have also noted, by directed enumeration of MART-1-specific TIL with epitope/HLA tetrameric complexes, that the frequency of MART-1-specific TIL is also not as high as predicted by those original studies [22]. Another commonly recognised antigen in the context of

HLA-A*0201 is gp100. Analysis of 217 fresh metastatic melanoma specimens demonstrated that, although the expression of MART-1 and gp100 *in vivo* can be quite heterogeneous, the majority of melanoma metastases express these TA [13]. Due to their frequency of expression, the main focus of vaccination efforts has been directed towards these two TA.

2.1 Peptide-based vaccines

Extensive analyses have shown that the MART-1:27-35 epitope is consistently recognised by MART-1-specific, tumour-recognising HLA-A*0201 restricted T-cells [23]. More than one HLA-A*0201 restricted peptide could be identified for the larger gp100 [24]. However, gp100 epitopes were not as efficient as MART-1:27-35 for *in vitro* induction of CTL. Thus, to enhance the immunogenicity of gp100 epitopes, single amino acid substitutions were made to increase the binding affinity to HLA-A*0201. A modified peptide was subsequently identified, gp100:209-217(210M) (g209-2M), with sequence IMDQVPFSV, modified from its natural sequence: ITDQVPFSV, with increased immunogenic potential *in vitro* and *in vivo* [25,26]. Since HLA-A*0201 is the predominant allele in the melanoma population [17], peptide-based vaccinations restricted to HLA-A*0201 patients were initiated by sc. administration of MART-1:27-35 emulsified in IFA. Comparison of reactivity of PBMC obtained before and after vaccination demonstrated strong enhancement of immune-competency toward MART-1 [27]. In a second trial, melanoma patients were treated with g209-2M alone or in combination with high dose iv. IL-2. Successful immunisation could be documented in patients immunised with g209-2M; however, no objective clinical responses were observed unless IL-2 treatment was added [28]. CTL has also been induced in patients receiving tyrosinase vaccination; in one study regression of a metastasis was noted in a patient with persistence of tyrosinase expression [29].

2.2 Dendritic cell-based vaccines

Preclinical studies suggest that the administration of peptide alone for the treatment of cancer is not as efficient as when an appropriate adjuvant was added [30]. Among adjuvants, dendritic cells (DC) play a critical role because they are highly specialised antigen-presenting cells (APC) with unique immunostimulatory properties. DC can induce primary cellular immune responses [31]. Activated DC can migrate from areas of antigen capture in the

peripheral tissues to areas infiltrated with naive T-cells, such as of lymphoid organs. DC have been shown also in preclinical models to play a role in class I-restricted antitumour sensitisation *in vivo* [32-38] and as an addition to immunisation with peptide alone [33,36,37]. These preclinical successes have led to peptide-pulsed DC-based clinical trials with varying results [39,40].

DC cultured in IL-4 and GM-CSF could sensitise *in vitro* PBMC from melanoma patients against HLA-A*0201-restricted epitopes, including MART-1:27-35 and g209-2M, by a single exposure of the responder cells to the relevant TA [41]. These findings stimulated a Phase I clinical trial at the National Cancer Institute (NCI) in which patients with metastatic melanoma were immunised with DC pulsed with MART-1 and gp100 epitopes. Previously reported clinical studies had generally used minimal numbers of DC compared with the potential yield of DC from a standard leukapheresis and had applied different routes of administration [39,40]. In the NCI study, up to 2×10^8 DC were safely administered intravenously. However, with the exception of one patient who experienced a temporary partial regression of sc. and pulmonary metastases, no clinical or immunological benefits were noted [13]. Nestle *et al.* reported high rates of tumour regression in response to the intra-lymphatic administration of a small number of DC prepared with methods similar to the those used in the NCI study and pulsed either with TA derived peptides or with tumour preparations [39]. Thurner *et al.* vaccinated eleven patients with MAGE-3A1 peptide-pulsed DC and was able to expand CTL in eight patients and reported clinical regression in six patients [42]. The different routes of DC administration, maturation status of DC and the addition of helper antigens including KLH and TT may be responsible for the significant differences in clinical outcomes of these DC trials.

2.3 Whole antigen vaccines

Usage of TA-derived peptides requires knowledge of the amino acid sequence of the epitope specific for each HLA allomorph [43]. Various strategies have been reported to obviate this problem. The use of acid-eluted peptides derived from autologous tumour has been proposed [55]. Other strategies have taken advantage of the ability of APC to incorporate exogenous particles or messenger-RNA (mRNA) and present them to T-cells [44,45]. Bhardwaj *et al.* [46] demonstrated that DC can be infected by viruses and

be permissive to the expression of viral products. We infected DC with viral constructs encoding TA to stimulate autologous human T-cells and observed that DC are permissive to poxvirus-driven expression of TA [41,47]. Virally-induced TA were naturally processed by DC and presented as relevant epitopes [47] which could efficiently induce TA-specific T-cells [41].

With virally-infected DC we extensively analysed the stringency of HLA allele/epitope requirements for immunogenicity of a particular antigen/HLA combination [48]. Those studies demonstrated that virally-induced TA-specific CTL by autologous DC is restricted to a unique allele/ligand combination and is excluded by minimal changes in HLA structure. Thus, the use of whole TA, including epitopes associated with multiple restriction elements, may not be as useful as theoretically predicted [49,50]. Adenoviral vectors have been tested in Phase I clinical trials. One of 16 patients receiving adeno-MART-1 experienced a complete clinical response with disappearance of all evaluable metastases. Other objective clinical responses occurred in patients receiving IL-2 simultaneously and thus could not be attributable specifically to the viral-TA vaccine. In addition, no consistent evidence of immunisation to MART-1 or gp100 could be demonstrated in contrast to the results described for peptide vaccines. It is possible that neutralising antibodies generated by the exposure to the virus eliminated the vector before it could generate antigen for immunisation [51].

2.4 DC/tumour hybrids

Some have advocated the construction of TA-presenting cell hybrids with the intent of combining the potency of DC as professional antigen-presenting cells with the unlimited antigenic potential of cancer cells. This approach has the obvious advantage of not requiring the molecular identification of all potential antigens expressed by each patient's cancer cells. Promising results were reported recently by hybridising DC with renal cancer cells [52]. Similarly, others attempted to hybridise autologous cancer cells to heterologous melanoma cells from established cell lines [53]. This approach has several advantages. It allows the expansion of autologous tumour cells in those cases when little starting material is available since the efficiency of the hybridisation is very high. In addition, the hybrids carry foreign HLA alleles that can induce a strong

immunologic reaction at the site of inoculation that may increase the potency of the vaccine.

3. Monitoring immune response and heterogeneity

3.1 Monitoring of anticancer vaccines

The ultimate goal of a vaccine is to promote cancer rejection. However, the biological goal is to increase the antitumour immune competence of the host. Antigen-specific vaccinations, although clinically disappointing, have given a unique opportunity to test localisation and activation status in the target organ of vaccine-specific systemic T-cell responses. At the same time, accurate documentation of the expression of molecules targeted by the vaccination can be performed. Thus, analysis of these molecular treatments allows the opportunity to directly measure immune responses that may in turn provide further insight into the biology of tumour rejection and suggest more effective methods of therapy.

To fully assess the effects of a cancer vaccine, laboratory studies are focused on monitoring the parameters of vaccine administration, biological properties and local or systemic effects. The assessment of patient and tumour-related factors suspected to influence antitumour immune response should be considered. It is essential to demonstrate that the vaccine induces an immune response that recognises the antigens as naturally processed and presented by tumour cells. Furthermore, assessment of the magnitude and quality of the immune response is likely to be important in determining the potential efficacy of the vaccine. For example, the number of TA-specific cells [54], their avidity for the antigen [55], the level and variety of cytokines they produce [56] and their tumour localisation may be critical for mediating antitumour effects [57].

Theoretically, the tumour is the most relevant tissue for monitoring immune responses, since lymphocytes or antibodies must reach the tumour in order to mediate antitumour effects. Examination of the tumour can also confirm that the relevant antigen and the associated HLA molecules are expressed. Post-vaccination sampling of tumour specimens can provide important information on immune selection. Even when this is feasible, an unsolved question is the timing of tissue sampling during vaccine administration. However, in most circumstances, it is only

possible to monitor specific responses in peripheral blood. This information verifies that the vaccine could induce a systemic immune response.

3.2 Patient heterogeneity in immune responses

Induction of an immune response may depend on patient-specific factors including prior treatment with cytotoxic agents, type and stage of disease, performance status etc. Some investigators have reported generalised or specific defects in T-cell signalling in patients with advanced cancer [58]. Defects in the maturation of professional APC due to tumour overproduction of vascular endothelial growth factor have also been reported [59]. Moreover, there are no reliable tests to evaluate immune competence and the ability to respond to antigen exposure. Many investigators rely only on general markers of immune responsiveness, such as delayed type hypersensitivity, CD4+/CD8+ ratios or lymphocyte responsiveness to mitogens, viral antigens or alloantigens. The relevance of these studies to assess antigen-specific immune competence, however, remains undetermined.

High resolution HLA typing is an important aspect of patient selection due to the HLA restriction of some vaccines [60]. Patients selected for treatment with a particular HLA-restricted peptide antigen must be shown to express that HLA molecule. Such a requirement may not be necessary when the immunising preparation is a whole protein or is derived from whole tumour cells. However, because of the difficulty in measuring and characterising immune responses to proteins or whole tumour cells, monitoring is often directed to specific HLA-restricted epitopic determinants of the proteins contained in the vaccine [51,61].

3.3 Tumour heterogeneity and immune response

Clinical data suggest the possibility of dissociation between immune responses detected in peripheral blood *versus* tumour [62]. Thus, assessment of tumour/host interactions at the tumour site can guide selection of appropriate patients for immunologic treatment and provide more accurate information about the biological basis for treatment failures and successes. Unfortunately, obtaining sufficient tumour tissue for these studies can be difficult, particularly when the tumours are not easily accessible. Furthermore, assays conducted on tumours are expensive and labour-intensive and interpretation of the results

is complicated by heterogeneity within and between tumour specimens.

Fine needle aspirates (FNA) of tumours, which can be performed with relative ease and minimal morbidity, can provide sufficient tissue for some correlative studies and give information on TA and HLA expression. In addition they can be used to establish both lymphocyte cultures and tumour cell lines for *in vitro* assays [63]. A number of other tumour-related factors have been identified that may determine responsiveness to a vaccine, including tumour expression of immunosuppressive cytokines and apoptotic signals or lack of expression of adhesion molecules on tumour vasculature that allow penetration by T-cells [64]. Due to the many factors that appear to impact on the antitumour immune response [65], newer technologies, such as gene chip arrays, which can measure expression of many genes simultaneously within a tumour tissue sample, may be necessary to fully characterise the tumour phenotype prior to immunisation [66].

3.4 Monitoring immune responsiveness at global transcript level

The identification of TA has provided a tool suitable for dissecting the molecular immunology of tumour/host interactions [67,68] by focusing on one single clearly defined target molecule at a time. However, as the resolution of our insight increases, new questions emerge regarding the natural history of immune-mediated adjustments of tumour phenotypes and, conversely, tumour-induced adaptation of the host immune competence [65,69]. As previously discussed, clinical studies have raised new questions based on the paradoxical observation that in several instances the induction of tumour-reactive circulating T-cells by the vaccine does not correlate with clinical effectiveness [27]. This discrepancy suggests that a clearly defined therapy against specific biological targets, which should lead to cancer rejection, is insufficient. Also, it represents a highly relevant example of the complexity of human cancer and the biological process underlying it. An extensive number of events downstream of the generation of tumour-reactive lymphocytes might explain the unpredictable behaviour of supposedly immune-responsive human cancers [65].

Understanding of the biological phenomena associated with tumour rejection in response to immune manipulation therefore will depend on new

technologies that allow for the global evaluation of thousands of gene interactions at one time [70]. We have paid particular attention to microarray technology, which allows comparative measurement of the expression of thousands of genes in relation to a biological process [71]. Among the various methods introduced, the utilisation of partial cDNA sequences from genes with known function or from expressed sequence tags (ESTs) from uncharacterised genes has been particularly successful [72]. These cDNA are spotted on a solid surface similar to a standard pathology slide. Total RNA or poly(A)-RNA from a tissue or cell line is then converted into cDNA, labelled with a reporter fluorescent molecule and then hybridised to the slide containing the arrayed genes. mRNA from a reference tissue or cell line is co-hybridised to the same slide after labelling with another fluorochrome. The addition of a consistent reference system allows correction for technical variability among hybridisations. Advances in computing power [73] and statistical tools [74] allow efficient interpretation of the extensive data derived by these analyses and allow discrimination between true expression patterns and random artefacts.

Advanced statistics can also characterise patterns of gene expression relevant to various biological processes [71,75,76]. Global patterns of gene expression could be obtained of neoplastic processes [77] and modulation of T-cell function [78]. Furthermore, 'portraits' of tumour specimens could be derived by the study of surgical specimens [79-81]. These efforts have identified molecular subsets of cancer based on mathematical analyses of their extended gene expression profile [77,79,80,82-84]. With the hope of identifying subcategories of disease with homogeneous behaviour, natural or treatment induced, Clark *et al.* identified several genes associated with metastatic potential in a murine model of human melanoma [85]. Bitner *et al.* described two biologically distinct molecular profiles of cutaneous melanoma with divergent metastatic potential *in vitro*, but due to limitations in the study design, no clinical correlates could be identified [80]. More recently, Perou *et al.* separated primary breast carcinomas into two groups with molecular portraits suggesting diversity in expression of markers potentially correlating with survival [81]. However, a direct association between the molecular profile of a solid tumour and its biological behaviour has not been as yet described. Our work suggests that melanoma metastases diverge biologically in 20 - 30% of patients

[13]. In addition, although Perou *et al.* [81] noted that primary breast tumours biopsied pre- and post-treatment do not change significantly during a short observation period, we and others have noted in metastatic melanoma that specific biological markers may vary significantly [62,86].

The difficulty of correlating laboratory findings with clinical outcome is a significant obstacle to the assessment of the role of immune escape and/or tolerance in cancer progression. Tumour/host interactions are generally studied in excised surgical specimens. These specimens, however, are not optimal for functional studies addressing the status of T-cell activation *in situ*. Freshly isolated tumour cells or lymphocytes are sub-optimal for accurate functional studies due to their extensive contamination by various cell types and the altered conditions of cells recently subjected to enzymatic or mechanical treatment. Expansion of TIL/tumour cell pairs provides elegant models for the characterisation *in vitro* of CTL/tumour interaction but these studies are only indirectly representative of *in vivo* conditions [87]. In addition, analysis of reagents obtained from excised specimens yields static information about a disease characterised by extreme genetic instability [88]. By excising the tumour mass, its natural or therapy-induced behaviour cannot be followed prospectively and an assumption is made perhaps erroneously that the excised lesion is representative of other lesions left *in vivo*. This limitation could be overcome by serial analyses of identical tumour samples through FNA, which provide the opportunity to evaluate dynamically the expression of relevant markers [12,62]. Due to the limited amount of material obtainable, however, FNA suffers from its own limitations. FNA could be combined with other techniques allowing analysis of limited materials. Distinct populations of cells could be sorted by micro-dissection [89,90] or epitope/HLA tetramers [91,92] and their status of activation could be tested using accurate and sensitive methodologies, such as Taqman-based real time RT-PCR [93] or intracellular FACS analysis [94]. This theoretically allows evaluation of the status of activation of CTL *in vivo*. Collection of cDNA libraries from FNA of metastases could profile patterns of expression of thousands of genes in a single experiment [66]. This information, combined with knowledge of the natural history of the lesion left *in situ*, might yield clinical material for the correlation of laboratory findings with clinical outcome and identification of the algorithm necessary for tumour

regression. Recent work in our laboratory has shown that the use of FNA-derived material may yield useful information about the kinetic of the interaction between the host immune system and cancer [63,95].

Since, of the increasing number of biological variables that could play a role in modulating tumour immune responsiveness, we believe that a global approach to the problem using microarray technology should be adopted. Although the *ex vivo* analysis of tissue samples using cDNA microarray technology is somewhat limited by the amount of RNA necessary for conventional cDNA microarrays (50 - 200 µg of total RNA or 2 - 5 µg poly(A)-RNA) corresponding to 106 - 107 cells sufficient for conventional microarray analysis. This number of cells is above the yield from FNA or micro-dissection specimens. To broaden the use of cDNA microarrays to experimental conditions in which source material is the limiting factor, we recently described a procedure that allows 105-fold enrichment of source RNA by combining antisense RNA (aRNA) amplification with template-switching effect to generate full-length double-stranded cDNA [96]. In this method, the amplified cDNA collection still retains the relative proportions of the original mRNA population and then is used as a target in cDNA microarrays. This allows analysis of global gene expression profiles in samples from FNA throughout the natural history of a tumour and/or in response to treatment. In addition, this technique might be utilised on micro-dissection specimens in which a finite number of cells (< 1000) are obtainable.

4. Assays of bulk cultures of peripheral lymphocytes

Immunisation against various antigens in animal models and humans has been shown to produce expansion of TA-specific T-cell precursors in PBMC. Comparative analysis of pre- and post-vaccination PBMC cultures is used to demonstrate expansion of vaccine-specific T-cells. Detection of T-cell reactivity in bulk cultures often requires repeated exposure of the T-cell precursors *in vitro* to the TA relevant to the vaccine in combination with IL-2 or other cytokines. Although lymphocytes can produce several cytokines, assays for detection of antigen-specific cells in bulk cultures are often designed to measure IFN-γ release upon antigenic stimulation or cytotoxicity of TA-bearing targets. By using these detection methods, successful immunisation to the MART-1

peptide was first demonstrated [27]. Characterisation of CTL responses in bulk culture can be expanded to estimate the avidity of the CTL for the vaccine. Detection of CTL activity at low E:T ratios, or recognition of peptide-pulsed target cells at very low concentrations of peptide, suggests that CTL have high avidity for the antigen-HLA complex [97]. Regardless of the methodology, the ultimate purpose of immune monitoring is to develop laboratory surrogate markers of clinical response.

4.1 ELISPOT assays

The ELISPOT assay was developed to provide quantitative evaluation of antigen-specific T-cell frequency [98]. Plates are coated with antibody to a specific cytokine. Target cells pulsed with peptide, or target tumour cells, are placed onto the plate, upon which the lymphocytes are added. Plates are incubated for approximately 24 - 48 h. Cytokines released by lymphocytes specific to the target will be captured by the pre-coated antibody and specifically detected by colourimetric assay. The cytokine spotted colonies are then counted and the total number of spots in plates loaded with the relevant target minus the spots formed in the plates with a non-relevant target is used to estimate the frequency of antigen-specific T-cells.

The published experience using the ELISPOT to monitor T-cell responses to cancer antigens is still limited [99]. ELISPOT assays conducted by Pass *et al.* in patients immunised with peptides derived from gp100 demonstrate that an 8 - 12 day *in vitro* sensitisation (IVS) with peptide and IL-2 was necessary to detect reactivity above background. After the IVS, peptide-reactive CTL could be detected in most patients immunised with the gp100:209-217(210M) modified peptide or the native gp100:209-217 peptide and in approximately 20% of patients immunised with the gp100:280-288 (288V) peptide. The frequency of CTL after the 8 - 12 day of IVS was in the range of 0.1 - 1%. When tumour was used as the target in the ELISPOT, reactive CTL at a frequency of 0.1 - 1% were demonstrated in several patients immunised with the modified gp100 peptide.

4.2 Limiting dilution assays

The limiting dilution assay (LDA) was also designed to yield an estimate of T-cell precursor frequency in the circulation and therefore to be a relatively quantitative assay. Furthermore, as expansion of T-cells is integral to the conduct of this assay, it has the capacity for increased sensitivity compared with assays, such as

ELISPOT, intracellular cytokine and tetramer analysis. The number of 'negative' cultures per dilution of the starting lymphocyte population can be plotted and statistical methods can be employed to determine the frequency of the target antigen-specific lymphocytes in the undiluted lymphocyte population obtained from peripheral blood [100]. Each culture per dilution is set up by adding feeder cells (usually irradiated PBMC) and then adding the antigen of interest (a peptide in many cases) or a tumour target. A parallel series of control cultures are established with irrelevant targets. The lymphocytes are added at a predetermined E:T ratio along with IL-2. After allowing a sufficient period of time for the antigen-specific lymphocytes to proliferate, labelled target cells containing the antigen are added to the cultures to test for specificity of each colony.

The results of the LDA depend on several variables including the type of cellular response that will be assayed, the target cells, the frequency of the antigen-specific cells in the starting population, the ability of those cells to expand in culture and the number of cultures established per dilution as well as the number of dilutions that are examined. Overall, the sensitivity of LDA appears to be superior to other assays that use a functional end point for detection of antigen-specific lymphocytes, with a range of 1/30,000 - 1/100,000. Even under the most favourable circumstances, however, measurement of lymphocyte responses with the LDA is labour-intensive and not practical for routine monitoring of cancer vaccine trials.

4.3 Intracellular cytokine production

A more direct assay quantifies TA-specific T-cells on the basis of intracellular cytokine production. T-cell membrane permeability is increased by chemical means, which allows penetration and binding by antibodies specific to a cytokine, usually IFN- γ . Multicolour flow cytometry can then be used to quantify and separate subsets of activated T-cells among a bulk PBMC population. The technique has not been fully developed nor used extensively to date in monitoring of cancer vaccine trials and is limited in overall by the sensitivity of flow cytometry [101].

4.4 Tetramer assays

Another direct assay enumerates *ex vivo* T-cells containing TCR capable of binding to a relevant peptide-MHC complex. When a lymphocyte TCR binds to only a single peptide-HLA complex with

relatively low affinity, it rapidly dissociates. However, when 2 - 4 HLA molecules are linked and the same peptide antigen is properly bound to the HLA molecules, the dissociation rate of is diminished substantially, allowing for identification and sorting of epitope-specific lymphocytes by FACS [91]. The sensitivity of peptide-HLA tetramers in detecting TA-specific CTL is greater than other direct functional assays, since the detection of T-cells is based primarily on the binding properties of the tetramer with the TCR. Indeed the only limit of detection is due to the sensitivity of the FACS instrument, which can reliably detect approximately 1/5000 - 1/10,000 cells (0.01 - 0.02%). The peptide-HLA tetramers provide reliable and direct measurements of TA-specific T-cells directly on peripheral blood or tissue. One major drawback to tetramer analysis, however, is the requirement for a defined epitope. Since melanoma is the only tumour type in which immunodominant putative TA are known, this type of analysis has exciting yet narrow applicability.

The published experience with tetramers to monitor CTL responses to cancer antigens remains limited [92,102]. Romero *et al.* demonstrated higher frequencies of MART-1/Melan-A CTL in CD8+ lymphocytes obtained from melanoma-involved regional lymph nodes. Lee *et al.* employed tetramers to tyrosinase, MART-1:27-35 and gp100:154-162 peptides to stain PBMC from 11 vaccine-naïve melanoma patients [103]. In four patients, MART-1-specific CTL were detected at a frequency of 0.014 - 0.16%, while tyrosinase-specific CTL were detected in two patients (0.19 and 2.2%). Based on a single patient analysis, these authors attempted to describe the status of activation of tumour reactive T-cells and concluded that the reason why cancer grows unaffected in humans is because T-cells that could potentially recognise tumour antigens are anergic. Such findings have been difficult to reproduce in studies when vaccine-induced T-cell reactivity was evaluated [101,104]. We used g209-2M tetramers to measure PBMC frequencies of vaccine-induced T-cells in patients immunised with this peptide [101]. While increases in CTL frequency could be detected in most post-immunisation PBMC, the absolute number of TA-specific T-cells reached a maximum of 1.0 - 1.5% of CD8+ T-cells. Tetramer staining appeared to correlate with other functional studies including quantitative real-time PCR and intracellular FACS analysis assessment of IFN- γ production in response to vaccine-related stimulation [104].

Several assays have been developed for measuring vaccine-induced T-lymphocyte responses. While each has advantages and disadvantages, none can measure the full spectrum of possible lymphocyte responses. In addition, there are no universally accepted correlates at this time between any method of *in vitro* immune monitoring and clinical outcome. For these reasons, these assays must be considered research techniques rather than fully validated instruments that can be used routinely to guide the development of cancer vaccines in the clinic. Monitoring becomes increasingly more directed as the antigen in the vaccine becomes more defined and restricted. In general, T-cell responses to vaccination include cell activation, changes in expression of surface markers, proliferation, cytokine production and antigen-specific lysis of target cells.

5. Functional analysis of TA-specific T-cells by gene expression

We recently developed molecular methods to estimate the presence, or changes in frequency, of T-cells in peripheral blood or tumour that have the activation characteristics in response to antigen recognition. Following activation of a T-cell, the mRNA for several cytokines and other activation-induced genes increases within 2–4 h as detected by quantitative RT-PCR reactions. The absolute copy number of mRNA for a particular gene and the increase in message related to T-cell activation can be measured. A sample of PBMC exposed to peptide antigen for different periods of time to can be used to study the kinetics of T-cell activation. Quantitative PCR for various cytokine genes is performed at several time points following the completion of peptide incubation [95,105].

Among several genes, IFN- γ appears to be the most sensitive for detecting activation of MHC class I-restricted CD8⁺ T-cells. The sensitivity of the assay, determined by spiking titrated amounts of cloned CTL to PBMC, can detect as few as one TA-specific T-cell among 50,000 PBMC. Based on preliminary studies of patients immunised with the g209-2M peptide, we believe that the sensitivity of quantitative PCR is below that of bulk *in vitro* culture of PBMC. Nevertheless the sensitivity of PCR may be sufficient for the purpose of detecting clinically relevant immune responses and the technique is substantially less labour-intensive. In addition, it may also be possible

to assess the avidity of the T-cell response by varying the concentration of peptide used to stimulate the T-cells.

6. *In vivo* biologic measures of T-lymphocyte responses

Regardless of the source of TA or method of immunisation, induction of an effective T-lymphocyte response against tumour should result in tumour infiltration by T-cells and other inflammatory effector cells drawn to the site by antigen-specific responses. Therefore, a direct method of monitoring vaccine efficacy is to obtain pre- and post-immunisation samples of tumour and assess the tumour inflammatory response. Optimally, the monitoring of T-cell responses within a tumour in response to a particular cancer vaccine would include assessment of the change in number of TA-specific cells, their activation state and functional properties. However, techniques capable of monitoring all aspects of the T-cell response are not yet available and the serial tumour biopsies necessary for these laboratory studies and full histologic examination are difficult to obtain, particularly in patients that have poorly accessible metastatic disease.

To address these concerns, we have adapted quantitative real time PCR to serially measure absolute amounts of RNA messages for genes expressed within samples of tumour obtained by FNA. While not able to ascribe changes to a particular group of antigen-specific T-cells, this technique can theoretically provide important information on changes in the overall amount of T-cell infiltration as well as the activation state and function of those cells. More importantly, the technique has the capacity to quantitatively measure biologic functions that are activated as part of a presumed final common pathway for antigen-specific T-cell mediated antitumour response. Preliminary experiments were conducted retrospectively on patients that had received g209-2M peptide vaccine [95]. In eight out of nine patients, post-treatment tumour samples revealed at least a 2-fold increase in copies of IFN- γ message; furthermore, the increase in IFN- γ mRNA correlated strongly to pre-vaccination tumour expression of gp100. Of substantial interest was the observation that none of the sampled lesions had shown evidence of objective response, suggesting that the immune response was insufficient to mediate tumour regression. The PCR

assay is currently being expanded to measure RNA message for other relevant genes, IL-2 for example.

7. Summary

The identification of TA has raised interest in the induction of specific activation and TA recognition by antigen-specific CTL. However, systemic T-cell responses to the vaccines often do not lead to objective clinical tumour regression. Among the questions raised by this paradoxical observation stands the enigma of whether tumour resistance to immunotherapy is due to insufficient immune response [106] or because tumour cells rapidly adapt to immune pressure by switching into less immunogenic phenotypes [65]. Of note, however, the vast majority of clinical trials were performed under less than optimal circumstances and often in patients with significantly advanced cancer. Furthermore, randomised Phase III trials with suitable control arms are only in the early stages. Despite the disappointment with epitope-specific vaccines, they have provided the unique opportunity of relating systemic T-cell responses with their localisation and status of activation in the target organ. It is likely that, in the future, combined analyses of systemic and intra-tumoural immune responses with new technologies described in this review will allow a more potent understanding of the algorithm governing tumour rejection by the immune system and lead to better vaccines with improved clinical responses.

Bibliography

Papers of special note have been highlighted as:

- of interest
- of considerable interest

1. YEWDELL JW, BENNINK JR: The binary logic of antigen processing and presentation to T-cells. *Cell* (1990) 62:203-206.
2. KNUTH A, DANOWSKI B, OETTGEN HF, OLD LJ: T-cell-mediated cytotoxicity against autologous malignant melanoma: Analysis with interleukin-2-dependent T-cell cultures. *Proc. Natl. Acad. Sci. USA* (1984) 81:3511-3515.
 - First demonstration of T-cell based recognition of cancer.
3. MARINCOLA FM, ROSENBERG SA: Biologic therapy with Interleukin-2. clinical applications: melanoma. In: *Biologic Therapy of Cancer*. DeVita VT, Hellman S, Rosenberg SA (Eds.) J. B. Lippincott Company, PA, USA (1995):250-262.
4. MC CE; MC CM: Systemic chemotherapy for the treatment of metastatic melanoma. *Semin. Oncol.* (1996) 23:744-753.
5. ROSENBERG SA: Keynote address: Perspective on the use of Interleukin-2 in cancer treatment. *Cancer J. Sci. Am.* (1997) 3:82-86.
6. ROSENBERG SA, LOTZE MT, YANG JC *et al.*: Prospective randomized trial of high-dose interleukin-2 alone or in conjunction with lymphokine-activated killer cells for the treatment of patients with advanced cancer. *J. Natl. Cancer Inst.* (1993) 85:622-632. [published erratum appears in *J. Natl. Cancer Inst.* (1993) 85:1091]
7. ROSENBERG SA, SPIESS P, LAFRENIERE R: A new approach to the adoptive immunotherapy of cancer with tumor-infiltrating lymphocytes. *Science* (1986) 233:1318-1321.
8. ITOH K, TILDEN AB, BALCH GM: Interleukin 2 activation of cytotoxic T-lymphocytes infiltrating into human metastatic melanomas. *Cancer Res.* (1986) 46:3011-3017.
9. KAWAKAMI Y, ZAKUT R, TOPALIAN SL, STOTTER H, ROSENBERG SA: Shared human melanoma antigens. Recognition by tumor-infiltrating lymphocytes in HLA-A2.1-transfected melanomas. *J. Immunol.* (1992) 148:638-643.
 - First comprehensive characterisation of the commonality of melanoma antigens.
10. VAN DER BRUGGEN P, TRAVERSARI C, CHOMEZ P *et al.*: A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* (1991) 254:1643-1647.
 - Identification of first melanoma antigen recognised by T-cells.
11. CHEN Y-T, STOCKERT E, TSANG S, COPLAN KE, OLD LJ: Immunophenotyping of melanomas for tyrosinase: Implications for vaccine development. *Proc. Natl. Acad. Sci. USA* (1995) 92:8125-8129.
12. MARINCOLA FM, HIJAZI YM, FETSCH P *et al.*: Analysis of expression of the melanoma associated antigens MART-1 and gp100 in metastatic melanoma cell lines and *in situ* lesions. *J. Immunother.* (1996) 19:192-205.
13. CORMIER JN, HIJAZI YM, ABATI A *et al.*: Heterogeneous expression of melanoma-associated antigens (MAA) and HLA-A2 in metastatic melanoma *in vitro*. *Int. J. Cancer* (1998) 75:517-524.
14. CORMIER JN, ABATI A, FETSCH P *et al.*: Comparative analysis of the *in vitro* expression of tyrosinase, MART-1/Melan-A and gp100 in metastatic melanoma lesions: implications for immunotherapy. *J. Immunother.* (1998) 21:27-31.
15. FETSCH PA, KLEINER D, MARINCOLA FM, ABATI A: Analysis of melanoma associated antigen MART-1 in normal tissues and in selected non-melanomatous neoplasms. *Modern Pathol.* (1997) 10:43.

16. CHEN YT, SCANLAN MJ, SAIBIN U *et al*: A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. *Proc. Natl. Acad. Sci. USA* (1997) 94:1914-1918.
- Identification of humoral responses directed against autologous antigens.
17. MARINCOLA FM, SHAMAMIAN P, RIVOLTINI L *et al*: HLA associations in the anti-tumor response against malignant melanoma. *J. Immunother.* (1996) 18:242-252.
18. PLAYER MA, BARRACCHINI KC, SIMONIS TB *et al*: Differences in frequency distribution of HLA-A2 sub-types between American and Italian Caucasian melanoma patients: Relevance for epitope specific vaccination protocols. *J. Immunother.* (1996) 19:357-363.
19. KAWAKAMI Y, ELIYAHU S, DELGADO CI *et al*: Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor. *Proc. Natl. Acad. Sci. USA* (1994) 91:3515-3519.
20. KAWAKAMI Y, ELIYAHU S, SAKAGUCHI K *et al*: Identification of the immunodominant peptides of the MART-1 human melanoma antigen recognized by the majority of HLA-A2- restricted tumor infiltrating lymphocytes. *J. Exp. Med.* (1994) 180:347-352.
21. KAWAKAMI Y, DANG N, WANG X *et al*: Recognition of shared melanoma antigens in association with major HLA-A alleles by tumor infiltrating lymphocytes from 123 patients with melanoma. *J. Immunother.* (2000) 23:17-27.
22. SPITER S, MONSURRO V, NIELSEN M-B *et al*: MART-1/melanoma and gp100/Pmel17 immunodominance revisited: Low frequency of melanoma differentiation antigen (MDA)-specific T-cells in tumor metastases. *J. Immunol.* (2000) (Submitted).
23. RIVOLTINI L, KAWAKAMI Y, SAKAGUCHI K *et al*: Induction of tumor reactive CTL from peripheral blood and tumor infiltrating lymphocytes of melanoma patients by *in vitro* stimulation with an immunodominant peptide of the human melanoma antigen MART-1. *J. Immunol.* (1995) 154:2257-2265.
- First demonstration that systemic immune responses can be raised using self tumour antigen-derived epitope.
24. SALGALLER ML, APSHAR A, MARINCOLA FM, RIVOLTINI L, KAWAKAMI Y, ROSENBERG SA: Recognition of multiple epitopes in the human melanoma antigen gp 100 by peripheral blood lymphocytes stimulated *in vitro* with synthetic peptides. *Cancer Res.* (1995) 55:4972-4979.
25. PARKHURST MR, SALGALLER ML, SOUTHWOOD S *et al*: Improved induction of melanoma reactive CTL with peptides from the melanoma antigen gp100 modified at HLA-A*0201 binding residues. *J. Immunol.* (1996) 157:2539-2548.
26. SALGALLER ML, MARINCOLA FM, CORMIER JN, ROSENBERG SA: Immunization against epitopes in the human melanoma antigen gp100 following patient immunization with synthetic peptides. *Cancer Res.* (1996) 56:4749-4757.
- One of the first demonstrations that a class I-restricted epitope alone can induce T-cell responses *in vivo*.
27. CORMIER JN, SALGALLER ML, PREVETTE T *et al*: Enhancement of cellular immunity in melanoma patients immunized with a peptide from MART-1/Melan A. *Cancer J. Sci. Am.* (1997) 3:37-44.
- One of the first demonstrations that a class I-restricted epitope alone can induce T-cell responses *in vivo*.
28. ROSENBERG SA, YANG JC, SCHWARTZENTRUBER D *et al*: Immunologic and therapeutic evaluation of a synthetic tumor associated peptide vaccine for the treatment of patients with metastatic melanoma. *Nature Med.* (1998) 4:321-327.
29. JAGER E, HINGHOFFER M, KARBACH U, ARAND M, OESCH F, KNUTH A: Inverse relationship of melanocyte differentiation antigen expression in melanoma tissues and CD8+ cytotoxic T-cell responses: evidence for immunoselection of antigen-loss variants *in vitro*. *Int. J. Cancer* (1996) 66:470-476.
30. VITTELLO A, ISHIOKA G, GREYDM *et al*: Development of a lipopeptide-based therapeutic vaccine to treat chronic HBV infection. I. Induction of a primary cytotoxic T lymphocyte response in humans. *J. Clin. Invest.* (1995) 95:341-349.
31. STENMAN RM: The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol.* (1991) 9:271-296.
32. PORGADOR A, SNYDER D, GILBOA E: Induction of antitumor immunity using bone marrow-generated dendritic cells. *J. Immunol.* (1996) 156:2918-2926.
33. CELLUZZI CM, MAYORDOMO JL, STORKUS WJ, LOTZE MT, FAO LD Jr: Peptide-pulsed dendritic cells induce antigen-specific CTL-mediated protective tumor immunity. *J. Exp. Med.* (1996) 183:283-287.
34. YOUNG JW, INABA K: Dendritic cells as adjuvants for class I major histocompatibility complex-restricted antitumor immunity (comment). *J. Exp. Med.* (1996) 183:7-11.
35. ZITVOGEL I, MAYORDOMO JL, TJANDRAWAN T *et al*: Therapy of murine tumors with tumor peptide-pulsed dendritic cells: dependence on T cells, B7 costimulation and T helper cell 1-associated cytokines (see comments). *J. Exp. Med.* (1996) 183:87-97.
36. PAGLIA P, CILIODONI C, RODOLFO M, COLOMBO MP: Murine dendritic cells loaded *in vitro* with soluble protein prime cytotoxic T lymphocytes against tumor antigen *in vivo* [see comments]. *J. Exp. Med.* (1996) 183:317-322.
37. MAYORDOMO JL, ZORINA T, STORKUS WJ *et al*: Bone marrow-derived dendritic cells pulsed with synthetic tumour peptides elicit protective and therapeutic antitumour immunity. *Nature Med.* (1995) 1:1297-1302.
38. FIELDS RC, SHIMIZU K, MUILE JJ: Murine dendritic cells pulsed with whole tumor lysates mediate potent antitumor immune responses *in vitro* and *in vivo*. *Proc. Natl. Acad. Sci. USA* (1996) 93:9482-9487.
39. NESTLE PO, ALJAGIC S, GILLIE M *et al*: Vaccination of melanoma patients with peptide or tumor lysate-pulsed dendritic cells. *Nature Med.* (1998) 4:328-332.

40. HISU FJ, BENIKE C, FAGNONI F *et al.*: Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. *Nature Med.* (1996) 2:52-58.
41. KIM CJ, FREVETTE T, CORMIER JN *et al.*: Dendritic cells infected with poxviruses encoding MART-1/MelanA sensitize T lymphocytes *in vitro*. *J. Immunother.* (1997) 20:276-286.
42. THURNER B, HARNDIE L, RÖDER C *et al.*: Vaccination with MAGE-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. *J. Exp. Med.* (1999) 190:1669-1678.
43. VAN DER BURG SIL, VISSEREN MJ, BRANDT RM, KAST WM, MELIEF JM: Immunogenicity of peptide bound to MHC class I molecules depends on the MHC-peptide complex stability. *J. Immunol.* (1996) 156:3308-3314.
44. HUANG AY, GOLIMBEK P, AHMADZADEH M, JAFFRE P, PARDOILL D, LEVITSKY IE: Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens. *Science* (1994) 264:961-965.
45. BOCKOWSKI D, NAIR SK, SNYDER D, GILBOA E: Dendritic cells pulsed with RNA are potent antigen-presenting cells *in vitro* and *in vivo*. *J. Exp. Med.* (1996) 184:465-472.
46. BIHARDWAJ N, BENDER A, GONZALEZ N, BUI LK, GARRETT MC, STEINMAN RM: Influenza virus-infected dendritic cells stimulate strong proliferative and cytolytic responses from human CD8⁺ T cells. *J. Clin. Invest.* (1994) 94:797-807.
47. KIM CJ, CORMIER JN, BODEN M *et al.*: Use of recombinant poxviruses to stimulate anti-melanoma T cell reactivity. *Ann. Surg. Oncol.* (1998) 5:64-76.
48. BETTINOTTI M, KIM CJ, LEE K-II *et al.*: Stringent allele/epitope requirements for MART-1/Melan A immunodominance: Implications for peptide-based immunotherapy. *J. Immunol.* (1998) 161:877-889.
49. SKOPPER JC, KITTLESSEN DJ, HENDRICKSON RC *et al.*: Shared epitopes for HLA-A3-restricted melanoma-reactive human CTL include a naturally processed epitope from Pmel-17/gp100. *J. Immunol.* (1996) 157:5027-5033.
50. YEE C, GILBERT MJ, RIDDELL SE *et al.*: Isolation of tyrosinase-specific CD8⁺ and CD4⁺ T cell clones from the peripheral blood of melanoma patients following *in vitro* stimulation with recombinant vaccinia virus. *J. Immunol.* (1996) 157:4079-4086.
51. ROSENBERG SA, ZHAI Y, YANG JC *et al.*: Immunization of patients with metastatic melanoma using recombinant adenoviruses encoding the MART-1 or gp100 melanoma antigens. *J. Natl. Cancer Inst.* (1998) 90:1894-1899.
52. KUGLER A, STÜHLER G, WALDEN P *et al.*: Regression of human metastatic renal cell carcinoma after vaccination with tumor cell-dendritic cell hybrids. *Nature Med.* (2000) 6:332-336.
53. NEWTON DA, ROMANO C, GATTONI-CELLI S: Semiallogenic cell hybrids as therapeutic vaccines for cancer. *J. Immunother.* (2000) 23:246-254.
54. SPIESER DE, MIRANDA R, ZAKARIAN A *et al.*: Self-antigens expressed by solid tumors do not efficiently stimulate naive or activated T cells: Implications for immunotherapy. *J. Exp. Med.* (1997) 186:645-653.
55. ZHOU H, PERRY-LALLEY D, DUDLEY ME, ROSENBERG SA, YANG J: High avidity CTLs for two self-antigens by solid tumors do not efficiently stimulate naive or activated T cells: Implications for immunotherapy. *J. Immunol.* (1999) 162:989-994.
56. BARTII RJ, MULE JJ, SPIESS P, ROSENBERG SA: Interferon gamma and tumor necrosis factor have a role in tumor regressions mediated by murine CD8⁺ tumor-infiltrating lymphocytes. *J. Exp. Med.* (1991) 173:647-658.
57. POCKAJ BA, SHERRY RM, WEI JP *et al.*: Localization of ¹¹¹Indium-labeled tumor infiltrating lymphocytes to tumor in patients receiving adoptive immunotherapy: Augmentation with cyclophosphamide and correlation with response. *Cancer* (1994) 73:1731-1737.
- Evidence that localisation of T-cells at the tumour site is necessary for tumour rejection.
58. MIZOGUCHI H, O'SHEA JJ, LONGO DL, LOEFFLER CM, MCVICAR DW, OCIOIA AC: Alterations in signal transduction molecules in T lymphocytes from tumor-bearing mice. *Science* (1992) 258:1795-1798.
59. GABRILOVICH DI, CHEN H, GIGIS KR *et al.*: Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. *Nature Med.* (1996) 2:1096-1103. [published erratum appears in *Nature Med.* (1996) 2(11):1267]
60. KIM CJ, PARKINSON DR, MARINCOLA FM: Immunodominance across the HLA polymorphism: Implications for cancer immunotherapy. *J. Immunother.* (1997) 21:1-16.
61. TSANG KY, ZAREMBA S, NIERODA CA, ZHU MZ, HAMILTON JM, SCHLOM J: Generation of human cytotoxic T cells specific for human carcinoembryonic antigen epitopes from patients immunized with recombinant vaccinia-CEA vaccine. *J. Natl. Cancer Inst.* (1995) 87:982-990.
62. LEE K-II, PANELLI MC, KIM CJ *et al.*: Functional dissociation between local and systemic immune response following peptide vaccination. *J. Immunol.* (1998) 161:4183-4194.
63. PANELLI MC, RIKER A, KAMMULA US *et al.*: Expansion of tumor/T cell pairs from fine needle aspirates (FNA) of melanoma metastases. *J. Immunol.* (2000) 164:495-504.
64. GANSS R, HANAUAN D: Tumor microenvironment can restrict the effectiveness of activated antitumor lymphocytes. *Cancer Res* (1998) 58:4673-4681.
65. MARINCOLA FM, JAFFE EM, HICKLIN DJ, PERRONE S: Escape of human solid tumors from T cell recognition: Molecular mechanisms and functional significance. *Adv. Immunol.* (2000) 74:181-273.

66. DUGGAN DJ, BITTNER M, CHIEN Y, MELTZER P, TRENT JM: **Expression profiling using cDNA microarrays.** *Nat Genet* (1999) **21**:10-14.
67. ROSENBERG SA: **Cancer vaccines based on the identification of genes encoding cancer regression antigens.** *Immunol. Today* (1997) **18**:175-182.
68. DRISCOLL MC, CHIU A, IHLGARTNER MW: **Heteroduplex analysis in hemophilia B: Detection of two novel Factor IX gene mutations.** *Am. J. Hematol.* (1996) **51**:324-327.
69. FUCHS EJ, MATZINGER P: **Is cancer dangerous to the immune system? *Semin. Immunol.* (1996) **8**:271-280.**
 - Application of 'danger model' to cancer immunology.
70. WANG E, MARINCOLA FM: **A natural history of melanoma: Serial gene expression analysis.** *Immunol. Today* (2000) (In press).
71. STAUDT LM, BROWN PO: **Genomic views of the immune system.** *Ann. Rev. Immunol.* (2000) **18**:829-859.
72. ADAMS MD, DUBNICK M, KEERLAVAGE AR *et al*: **Sequence identification of 2,375 human brain genes.** *Nature* (1992) **355**:632-634.
73. BUTLER D: **Computing 2010: from black holes to biology.** *Nature* (1999) **402**:C67-70.
74. CHIEN Y, DOUGHERTY ER, BITTNER ML: **Ratio-based decisions and the quantitative analysis of cDNA microarray images.** *Biomedical Optics* (1997) **2**:364-374.
75. SCIERNA M, SIALON D, DAVIS RW, BROWN PO: **Quantitative monitoring of gene expression patterns with a complementary DNA microarray.** *Science* (1995) **270**:467-470.
76. EISEN MB, SPELLMAN PT, BROWN PO, ROTSTEIN D: **Cluster analysis and display of genome-wide expression patterns.** *Proc. Natl. Acad. Sci. USA* (1998) **95**:14863-14868.
77. DERISI J, PENLAND T, BROWN PO *et al*: **Use of cDNA microarray to analyze gene expression patterns in human cancer.** *Nat. Genet.* (1996) **14**:457-460.
78. ALIZADEH AA, EISEN MB, ROTSTEIN D, BROWN PO, STAUDT LM: **Probing lymphocyte biology by genome-scale gene expression analysis.** *J. Clin. Invest.* (1998) **101**:373-379.
- Molecular characterisation of cancer based on extended gene expression profiling.
79. PEROU CM, JEFFREY SS, VAN DE RUIJ M *et al*: **Distinctive gene expression patterns in human mammary epithelial cells and breast cancers.** *Proc. Natl. Acad. Sci. USA* (1999) **96**:9212-9217.
- Molecular characterisation of cancer based on extended gene expression profiling.
80. BITTNER M, MELTZER P, CHIEN Y *et al*: **Molecular classification of cutaneous malignant melanoma by gene expression: Shifting from a continuous spectrum to distinct biologic entities.** *Nature* (2000) **406**:536-540.
- Molecular characterisation of cancer based on extended gene expression profiling.
81. PEROU CM, SERTLE T, EISEN MB *et al*: **Molecular portraits of human breast tumors.** *Nature* (2000) **406**:747-752.
- Molecular characterisation of cancer based on extended gene expression profiling.
82. GOLUB TR, SLONIM DK, TAMAYO P *et al*: **Molecular classification of cancer: Class discovery and class prediction by gene expression monitoring.** *Science* (1999) **286**:531-537.
83. KHAN J, SIMON R, BITTNER M *et al*: **Gene expression profiling of alveolar rhabdomyosarcoma with cDNA microarrays.** *Cancer Res.* (1998) **58**:5009-5013.
84. ALIZADEH AA, EISEN MB, DAVIS RE *et al*: **Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling.** *Nature* (2000) **403**:467-478.
85. CLARK EA, GOLUB TR, LANDER ES, HYNES RO: **Genomic analysis of metastasis reveals an essential role for RhoC.** *Nature* (2000) **406**:532-535.
86. JAGER E, RINGHOFFER M, ALTMANN-SCHNEIDER M *et al*: **Immunoselection *in vivo* independent loss of MHC class I and melanocyte differentiation antigen expression in metastatic melanoma.** *Int. J. Cancer* (1997) **71**:142-147.
87. PANDOLFI F, BOYLE LA, TRENTIN I, KURNICK JT, ISSELBACHER KJ, GATTIONI-CELLI S: **Expression of HLA-A2 antigen in human melanoma cell lines and its role in T-cell recognition.** *Cancer Res.* (1991) **51**:3164-3170.
88. LENGHAUER C, KINZLER KW, VOGELSTEIN B: **Genetic instabilities in human cancers.** *Nature* (1998) **396**:643-649.
89. BONNER RP, EMMERT-BUCK M, COLE K *et al*: **Laser capture microdissection: molecular analysis of tissue.** *Science* (1997) **278**:1481-1483.
- New method to separate cell populations *ex vivo*.
90. PETERSON LA, BROWN MR, CARLISLE AJ *et al*: **An improved method for construction of directionally cloned cDNA libraries from microdissected cells.** *Cancer Res.* (1998) **58**:5326-5328.
91. ALTMAN JD, MOSS FI, GOULDER PR *et al*: **Phenotypic analysis of antigen-specific T lymphocytes.** *Science* (1996) **274**:94-96 [published erratum appears in *Science* (1998) **280**:1821].
- New technology allowing immune phenotyping of antigen-specific T-cells.
92. ROMERO P, DUNBAR PR, VALMORI D *et al*: **Ex vivo staining of metastatic lymph nodes by class I major histocompatibility complex tetramers reveals high numbers of antigen-experienced tumor-specific cytolytic T lymphocytes.** *J. Exp. Med.* (1998) **188**:1641-1650.
93. KRUSE N, PETTE M, TOYKA K, RIECKMANN P: **Quantification of cytokine mRNA expression by RT-PCR in samples of previously frozen blood.** *J. Immunol. Methods* (1997) **210**:195-203.

94. KERN P, SUREL IP, BROCK C *et al*: **T-cell epitope mapping by flow cytometry.** *Nature Med.* (1998) 4:975-978.
95. KAMMULA US, LEE K-II, RIKER A *et al*: **Functional analysis of antigen-specific T lymphocytes by serial measurement of gene expression in peripheral blood mononuclear cells and tumor specimens.** *J. Immunol.* (1999) 163:6667-6679.
96. WANG E, MILLER L, O'INMACIIT GA, LIU E, MARINCOLA FM: **High fidelity mRNA amplification for gene profiling using cDNA microarrays.** *Nature Biotech.* (2000) 17:457-459.
- New method allowing utilisation of limited tissue material for extensive molecular analysis.
97. DUDLEY ME, NISHIMURA MI, HOLT AKC, ROSENBERG SA: **Anti-tumor immunization with a minimal peptide epitope (G9-209-2M) leads to a functionally heterogeneous CTL response.** *J. Immunother.* (1999) 22:288-298.
98. SCHEIBENBOGEN C, LEE KII, STEVANOVIC S *et al*: **Analysis of the T cell response to tumor and viral peptide antigens by an IFN- γ -ELISPOT assay.** *Int. J. Cancer* (1997) 71:932-936.
99. PASS HA, SCHWARZ SL, WUNDERLICH JR, ROSENBERG SA: **Immunization of patients with melanoma peptide vaccines: immunologic assessment using the ELISPOT assay.** *Cancer J. Sci. Am.* (1998) 4:316-323.
100. COULIE PG, SOMVILLE M, LEHMANN F *et al*: **Precursor frequency analysis of human cytolytic T lymphocytes directed against autologous melanoma cells.** *Int. J. Cancer* (1992) 50:289-297.
101. LEE K-II, WANG E, NIELSEN M-B *et al*: **Increased vaccine-specific T cell frequency after peptide-based vaccination correlates with increased susceptibility to *in vitro* stimulation but does not lead to tumor regression.** *J. Immunol.* (1999) 163:6292-6300.
102. OGG GS, ROD DP, ROMERO P, CHEN JL, CERUNDOLO V: **High frequency of skin-homing melanocyte-specific cytotoxic T lymphocytes in autoimmune vitiligo.** *J. Exp. Med.* (1998) 188:1203-1208.
103. LEE PP, YEE C, SAVAGE PA *et al*: **Characterization of circulating T cells specific for tumor-associated antigens in melanoma patients.** *Nature Med.* (1999) 5:677-685.
104. NIELSEN M-B, MONSURRO V, MIGUELSE S *et al*: **Status of activation of circulating vaccine-elicited CD8⁺ T cells.** *J. Immunol.* (2000) 165:2287-2296.
105. KAMMULA US, MARINCOLA FM, ROSENBERG SA: **Real-time quantitative polymerase chain reaction assessment of immune reactivity in melanoma patients after tumor peptide vaccination.** *J. Natl. Cancer Inst.* (2000) 92:1336-1344.
106. MATZINGER P: **An innate sense of danger.** *Semin. Immunol.* (1998) 10:399-415.

Ena Wang, Gao Q Phan & Francesco M Maincola[†]

[†] Author for correspondence

Surgery Branch, National Cancer Institute, National Institutes of Health Building 10, Room 2B42, 9000 Rockville Pike, Bethesda, MD 20892, USA

Tel.: +1 301 496 3098; Fax: +1 301 402 0922;

E-mail: maincola@nih.gov

Human Tumor Xenografts in the Nude Mouse and their Value as Test Models in Anticancer Drug Development (Review)

B. WINOGRAD¹, E. BOVEN², M.W. LOBBEZOO¹, and H.M. PINEDO^{1,2}

¹EORTC New Drug Development Office;

²Department of Oncology, Free University Hospital, De Boelelaan 1117, NL-1081 HV Amsterdam, The Netherlands

Abstract. *In an attempt to increase the predictability of preclinical antitumor testing, the value of human tumor lines in immune-deficient nude mice is assessed by reviewing the relevant literature. This test model is rather elaborate due to the nature of the animal as well as test and evaluation procedure. However, it represents a realistic simulation of clinical drug treatment. This is demonstrated by (a) a good correlation of drug effects in the nude mouse with clinical results in the donating patient's tumor and (b) by a good predictability of a panel of human tumor lines for clinically effective drugs. In order to avoid clinical trials with inactive drugs and no therapeutic benefit for a large number of patients, the application of human tumor xenografts in anticancer drug development is warranted.*

The development of new drugs for the treatment of cancer is a stepwise process leading from the isolation or the synthesis of a product to its general use in medical practice (figure 1). Natural or synthetic products are taken through the various stages in order to evaluate whether they can improve the treatment results of malignant disease.

At the screening level, a product is systematically investi-

gated for activity in a series of test models with a proven or presumed predictability for therapeutic effects in cancer. Generally only a very small proportion of the products investigated will fulfill the pre-set criteria for activity at the screening stage. A drug with presumed anticancer activity has to be produced on a large scale, and an acceptable formulation for clinical application has to be developed. The new drug must be evaluated for possible side effects at the toxicology stage. When there is a favourable balance between therapeutic and toxic effects the drug may enter clinical trials.

A new drug with potential activity against cancer will enter phase I clinical trials. These studies have been set up to determine the maximum tolerated dose that can be given to patients without unacceptable side effects and the type of dose - limiting toxicity for a given treatment schedule. Generally phase I studies are relatively short-lasting (0.5 - 1 year) and require a limited number of patients (20 - 100). On the basis of these results, phase II studies are initiated in order to assess the activity of the new drug on a variety of tumor types - its clinical antitumor profile. Usually 14 - 20 fully evaluable patients must be included for each tumor type. Only if significant activity is observed phase III studies may be performed to compare the effects of the new drug with those of established drugs in responsive tumor types. Phase II and III studies are generally long - lasting and involve a large number of patients. The new drug may be introduced into general practice when favourable treatment effects or reduced toxicity have been demonstrated compared to conventional anticancer drugs. After each step of anticancer drug development, a decision must be taken as to whether the product is suitable to proceed to the next step.

Unfortunately, many new drugs developed over the past years appeared to be active in only a few tumor types or were totally inactive. Thus a large proportion of phase II trials concluded with a negative result. One would prefer to select tumor types for phase II studies on the basis of a preclinical antitumor profile. If a preclinical screening method which could reliably predict the activity of a new drug in a particular tumor type were available, this would enable a more rational

Abbreviations: BCNU = 1,3-bis-(2-chloroethyl)-1-nitrosourea; CCNU = 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; CDDP = cisplatin; CTX = cyclophosphamide; CR = complete response; DTIC = dacarbazine; DXR = doxorubicin; 5-FU = 5-fluorouracil; GD = growth delay; HMM = hexamethylmelamine; MeCCNU = methyl CCNU; MTW T/C % = mean tumor weight inhibition in % treated / control; MTX = methotrexate; NSCLC = non-small cell lung carcinoma; PR = partial response (> 50% tumor volume reduction); SCLC = small cell lung carcinoma; SGD = specific growth delay; STS = soft tissue sarcoma; VCR = vincristine.

Correspondence to: Dr. B. Winograd.

Key Words: Tumor xenografts, human, nude mouse, anticancer drug development.

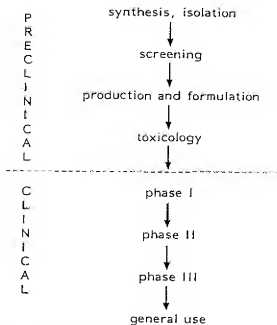


Figure 1. Stages of new drug development.

selection of tumor types for phase II studies. In addition, the number of patients treated with inactive agents might also be reduced.

In this paper we shall analyse the screening step presently in use for anticancer drug development. In an attempt to increase the predictability of antitumor testing, we propose the wide application of human tumor lines in immunodeficient nude mice. The biology of the nude mouse will be described and the establishment and the behaviour of human tumors transplanted in these animals will be reviewed. The value of human tumor lines for chemotherapy purposes will be analysed by assessing their predictability for both the individual patient's response and the responsiveness of specific tumor types. Finally, the advantages and drawbacks of this novel approach in the screening of drugs will be presented, aiming at a better prediction of sensitive tumor types in phase II clinical trials.

Screening in new drug development

The purpose of screening is twofold: to select active agents from a set of products and to determine the profile of activity of these drugs.

The selection of active drugs from a usually large number of products is frequently done by means of relatively simple test models and often using only one model: the primary screen or prescreen. Drugs shown by the primary screen to be active are then subjected to further screening and profiling in a secondary screen, mostly consisting of a series or a panel of more sophisticated test models. Different dose schedules and routes of administration may also be included here. After

completion of the screening step one can distinguish between those compounds that meet the activity criteria of the total screen (positives) and those that do not meet these criteria (negatives).

A further subdivision can be made after clinical evaluation:

- True positives: actives in the screening stage for which clinical activity is proven.
- False positives: actives in the screening stage which do not have any clinical activity.
- True negatives: inactives in the screening stage which do not have any clinical activity.
- False negatives: inactives in the screening stage for which clinical activity is proven.

The incidence of true negatives and false negatives is often not well known, as negative agents will not be evaluated clinically unless there are other data, e.g. from test models not incorporated in the screen, which lead to a "by-passing" of the screen. Drugs that proved inactive in the clinic are, however, very useful for validating new screening methods. These new methods should indicate false positives from previous screening models to be negatives.

The choice of the primary screening model is critical for the final outcome of drug development. The need to test large numbers of compounds in the primary screen has favoured the use of relatively fast, simple and cheap models such as *in vitro* techniques. *In vitro* models clearly increase the risk of false positives (e.g. metabolically unstable drugs) and false negatives (e.g. agents requiring bioactivation *in vivo*). The bias of a primary screen should always be taken into account and certain shortcomings may be compensated for by the design of the subsequent secondary screen.

The definition of criteria for distinguishing between actives and inactives is another critical issue involved in primary screening. These criteria should be closely related to the desired therapeutic effect in the clinic. Testing of a representative set of clinically active and inactive drugs in the primary screen could help in the establishment of a meaningful cut-off level of activity.

The number of compounds in the secondary screen will be only a fraction of those in the primary screen, and therefore more elaborate test models can be employed. Such models may provide a more realistic representation of the clinical situation. Defining stringent criteria for activity in the secondary screen may render it easier to predict a compound's clinical efficacy.

Screening for new anticancer drugs

In 1955 the American National Cancer Institute (NCI) started an acquisition and screening program in the search for new anticancer drugs. Since then, the NCI has become the largest single institution involved in the discovery and development of such drugs. The NCI program underwent major modifications in 1975 as a result of the experience gained in

Figur

the
and
NCI
form
ties:
comj
plac
pres
and
for
muri
from
pane
graft
the P
the J
bioio
The
depk
charz
Th
scre
test
drug
two
in 1975,
mammary
because
responses
lines (bre
CX-1) we
in nude m
for clinical

CURRENT DCT STANDARD SCREEN

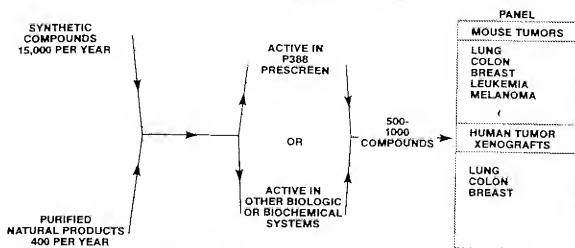


Figure 2. Flow of drugs through the standard screen of the NCI (from ref. 4).

the preceding years. From 1975 to 1985 the NCI acquisition and screening program has been as follows. Each year, the NCI acquired 20,000 - 40,000 new synthetic compounds, fermentation and natural products from industries, universities and other institutes, both in the USA and abroad. Before compounds entered the screening stage, preselection took place based upon a computer rating of structure novelty, presumed activity in the prescreen and a structure-activity analysis (1, 2). The 10,000 - 20,000 products finally selected for screening were entered into the prescreen, the P388 murine lymphocytic leukemia grown in normal mice. Actives from the P388 test system proceeded into a standard tumor panel, consisting of murine tumors and human tumor xenografts (2-4). New products lacking even minimal activity in the P388 prescreen were usually rejected for further testing in the panel, unless they showed activity in other relevant biologic or biochemical test systems ("bypass" compounds). The flow of compounds through the NCI standard screen is depicted in figure 2 and the tumor models employed are characterized in Table I.

The P388 murine leukemia was chosen as the primary screen because it was found to be the single most sensitive test detecting a large number of clinically known anticancer drugs (4). Compared to screening during the preceding years, two new aspects were incorporated into the NCI tumor panel in 1975. Two murine solid tumors (colon cancer 38 and mammary tumor CD8F1) grown subcutaneously were added, because it seemed probable that these would predict drug responses in solid tumors in man (5, 6). Three human tumor lines (breast cancer MX-1, lung cancer LX-1, colon cancer CX-1) were also added and used as subcutaneous transplants in nude mice. These tumor models were expected to predict for clinical activity of drugs in the three most frequent tumor

types. In the past few years these lines have been employed mainly in short-term assays using tumor transplants under the renal capsule of immune-deficient mice (7).

In spite of these changes, clinical evaluation of new drugs showed that the NCI standard screen has continued to result in a low level of prediction (8) and has defined only a few new anticancer drugs. It can be concluded that:

- most of the clinically effective anticancer drugs were active against selected tumor types, mainly leukemias and lymphomas;

- many false positive compounds have emerged and were evaluated in numerous clinical trials; many patients received "treatment" with inactive agents.

The extent to which the standard screen has yielded false negatives can of course only be answered partially. 2'-Deoxycoformycin (NSC 218321) is devoid of antitumor activity in the NCI standard screen but has shown significant efficacy in clinical practice (9, 10).

Several explanations may account for the low level of prediction by this standard screen:

- The choice of the prescreen model P388, a murine leukemia with a short tumor doubling time, may have supported the selection of new drugs in a disease-oriented fashion.

- The addition of murine solid tumors to the panel does not seem to have significantly improved the yield of this screening. In this respect it should be mentioned that chemotherapy experiments in murine tumor models are markedly different from the clinical situation. There are differences in tumor doubling time, tumor burden at the start of treatment, the applied drug dose and the methods of evaluation of tumor response between mice and man (11, 12). Also, incorporation of the three human tumor lines has not greatly

Table 1. Experimental tumor models employed for antitumor testing by the NCI (3,4).

Tumor	Origin	Mouse Strain	Inoculum	Site	Parameter	Activity criteria*
P388 leukemia	murine	CDF ₁ or BDF ₁	30 ⁶ cells ascites	i.p.	median survival time	T/C 120%
L1210 leukemia	murine	CDF ₁ or BDF ₁	10 ⁶ cells ascites	i.p.	median survival time	T/C 125%
B16 melanoma	murine	BDF ₁ or B6C3	1:10 homo-genate	i.p.	median survival time	T/C 125%
Lewis lung carcinoma	murine	BDF ₁	10 ⁷ viable cells	i.v.	median survival time	T/C 140%
Colon 26 ^b	murine	CDF ₁	1% brof	i.p.	median survival time	T/C 130%
Colon 38	murine	BDF ₁	fragment	s.c.	tumor weight inhibition	T/C 42%
CD8F ₁ mammary tumor	murine	CD8F ₁	5 × 10 ⁵ cells	s.c.	tumor weight inhibition	T/C 42%
MX-1 mammary xenograft	human	nu/nu Swiss	fragment	s.c.	tumor weight inhibition	T/C 42%
LX-1 lung xenograft	human	nu/nu Swiss	fragment	s.c.	tumor weight inhibition	T/C 20%
CX-1 lung xenograft	human	nu/nu Swiss	fragment	s.c.	tumor weight inhibition	T/C 20%
Colon xenograft	human	nu/nu Swiss	fragment	s.c.	tumor weight inhibition	T/C 20%

*observed parameter in treated animals / control animals × 100.

^bonly employed for special comparisons^cs.r.c.: subrenal capsule.

improved the predictive value of the standard screen. It should be taken into account that CX-1 and LX-1 are lines from tumor types which are clinically unresponsive to chemotherapy. This is exemplified by their low chemosensitivity to standard chemotherapeutics in the subcutaneous assay, 0/20 for CX-1 and 1/20 for LX-1 (7).

There is obviously a great need for better, more predictive experimental test models for anticancer drugs. Since 1985 the NCI has largely reduced its screening efforts in the standard screen and has concentrated on the development of a new disease-oriented screening system *in vitro*, which aims at employing a panel of *in vitro* human tumor cell lines per tumor type as a primary screen (13). Another novel, thus far very promising, approach in the screening of new anticancer drugs is the use of human tumor lines in nude mice, which will be discussed in the following sections.

The nude mouse

Immune-deficient mice have been used increasingly in cancer research over the past years. Both genetically immune-deficient mice and artificially immune-deprived mice have been described. Normal mice with artificially induced immune deficiency, (e.g. by combined treatment of thymectomy, cytosine arabinoside i.p. followed by whole-body irradiation), will not be discussed here. These mice generally have an unstable immune response and recover the ability to reject foreign tissue within a few months following this procedure (14).

The nude mouse is a hairless mutant first described in 1962 (15). This mouse appears to be homozygous for an autosomal recessive gene, genetic symbol *nu* (16). The animals are genetically immune deficient, because they lack a normal thymus. The mice therefore have a low number of T-lymphocytes but they have a well-developed B-lymphocyte system (17-19). The animals show impaired growth and a low fertility. Without proper precautions they usually die from infections before four months of age. The reduced cell-mediated immune reaction accounts for an enhanced sensitivity to viral infections, particularly by the murine hepatitis virus and the Sendai virus (18, 20, 21). Importantly, the deficiency in the cell-mediated immune response in nude mice is extremely useful for experiments with foreign tissue transplants, such as human tumor tissue (22, 23). However, viral and also bacterial infections not only affect the general condition of the nude mouse but also the growth of the transplanted human tumor. The reason for this growth inhibition or even tissue rejection may be the continuous activation of the residual immune system of the animals, causing an increase in both natural-killer cell activity and number of activated macrophages (24-27).

The use of nude mice in experimental cancer research requires special facilities and well-trained technicians. Detailed guidelines have been issued by the "Committee on care and use of the nude mouse in biomedical research" (28). These facilities include maintenance in isolation from normal animals, filtering of air, a high ambient temperature and humidity, sterilisation of cages, bedding, food and water, and

a: Fragment
transcutable
b: Take rate
c: Percentage
d: n.s.: not stable

the use of sterile
the mice can
conditions, a life
can be reached

Table II. Growth of human tumor types transplanted subcutaneously into nude mice.

Tumor type	Inoculation procedure ^a	Attempts number	Take rate ^b	Serial transfer ^{b,c}	Ref
Gastric cancer	fragments	49	43	16	38
Gastric cancer	fragments	44	68	39	39
Gastric cancer	fragments	33	46	27	40
Gastric cancer	fragments	9	22	11	41
Esophageal cancer	fragments	6	83	67	42
Gastro-intestinal	suspension	29	30	15	39
Colorectal cancer	fragments	83	78	55	39
Colorectal cancer	suspension	18	72	28	43
Colorectal cancer	fragments	9	67	56	44
Melanoma	suspension	39	67	44	23
Melanoma	fragments	26	39	27	45
Melanoma	fragments	19	89	42	39
Melanoma	suspension	9	56	56	42
Melanoma (eye)	fragments	6	67	n.s. ^d	45
Lung cancer	suspension	47	51	11	43
Lung cancer (NSCLC)	fragments	44	81	61	39
Lung cancer	suspension	22	50	31	46
Lung cancer	fragments	17	53	29	41
Lung cancer (SCLC)	suspension	29	45	31	47
Lung cancer (SCLC)	fragments	6	83	50	39
Head and neck cancer	suspension	25	36	12	43
Head and neck cancer	fragments	117	30	9	48
Kidney cancer	fragments	17	59	35	39
Kidney cancer	suspension	11	46	46	42
Wilm's tumor	suspension	18	56	56	49
Wilm's tumor	suspension	9	33	33	50
Lower urinary tract	suspension	8	13	13	42
Primitive germ cell	suspension	19	32	21	42
Testis cancer	fragments	11	36	18	39
Breast cancer	suspension	127	53	5	22
Breast cancer	suspension	87	16	5	43
Breast cancer	fragments	9	0	0	41
Ovarian cancer	fragments	68	27	n.s.	51
Ovarian cancer	fragments	44	32	11	52
Ovarian cancer	fragments	23	57	26	53
Ovarian cancer	fragments	15	67	20	54
Sarcoma	suspension	62	52	n.s.	54a
Sarcoma	suspension	23	70	39	23
Sarcoma (STS)	fragments	16	75	56	39
Sarcoma (bone)	suspension	14	71	71	50
Neuroblastoma	suspension	22	32	32	50
Lymphoid cancers	suspension	11	18	18	50
Unknown primary	suspension	20	45	25	42
Various cancers	suspension	88	51	28	23
Various cancers	fragments	32	68	50	55

a: Fragments are small pieces of solid tumor tissue implanted through a skin incision, while a suspension contains finely minced tumor tissue injected subcutaneously.

b: Take rate and serial transfer as percentage of attempts.

c: Percentages indicate the success of ≥ 2 subpassages.

d: n.s.: not stated.

the use of sterile attire by personnel handling the animals. If the mice can be maintained under specific pathogen-free conditions, a life span of normal mice (approximately 2 years) can be reached (28).

Human tumor lines

This paper will be confined to the use of human tumor lines as subcutaneous xenografts in nude mice. Other sites of implantation

tation, especially under the kidney capsule, have been frequently employed in drug testing (29). This subrenal capsule assay can be performed both in normal immune-competent mice (30-33) as well as in nude mice (34). A major disadvantage of this method is the difficulty in observing the growth of the implanted tumor fragment during the course of the assay, which thus reduces its attractiveness for chemotherapy experiments. Further limitations are the tissue rejection preceded by infiltration of mononuclear cells if normal mice are being used (35-37). Experience with chemotherapy in the subrenal capsule assay performed in immune-deficient mice, including nude mice, is still limited (34).

Methodology. Since the first successful transplantation of human colon cancer tissue into the nude mouse (22), many attempts to grow human tumors subcutaneously (s.c.) in these animals have been reported. Once an s.c. implanted tumor fragment has been accepted, the proliferating nodule can be excised and transplanted into the next series of recipients. By repeated serial transfer a tumor line may be established; this is necessary in order to ensure its continuous use in a long-term research program, such as the development of new drugs.

Specimens of primary tumors or metastases are obtained at surgery or diagnostic biopsy. These samples are transferred to a suitable medium (e.g. Hanks' balanced salt solution) and rapidly processed in the laboratory. Tumor fragments of 2 to 3 mm diameter or a tumor tissue suspension are implanted subcutaneously in the flanks of the animals. If the xenograft is not rejected, the tumor cells start to proliferate and a nodule will become palpable after a latency period of one to several weeks. Progressive growth to at least 50 mm³ is recorded as a "take". Larger nodules will be used for serial transfer. As illustrated in Table II, the initial take rate and the serial transplantability may vary considerably. This variability can be partially explained by differences between tumor types. Examples of easily transplantable tumors are colorectal cancer, melanoma, sarcoma and lung cancer, while breast cancer, ovarian cancer and cancer of the head and neck region are difficult to grow in nude mice. Other variables influencing the take rate of tissue transplants are factors related to the host; its general condition is the most important of these. In contrast to tumors in patients, metastases of human tumor xenografts are rarely found in nude mice (43).

Characterization. Once a human tumor line has been established, it should be monitored frequently to guarantee the retention of original characteristics such as macroscopic growth characteristics expressed by the latency period and the tumor volume doubling time. Both parameters tend to decrease during the first few serial passages, as has been demonstrated for a variety of lines derived from head-and-neck cancer (56), breast cancer (57), melanoma (58), pediatric tumors (49, 50), colorectal cancer (59) and gastrointestinal cancer (60). After the third or fourth serial passage in the

mouse a tumor line usually maintains a consistent doubling time.

Histological and immunohistochemical characterization of the xenograft tissue is essential in order to demonstrate its identity with or close similarity to the original tumor tissue. Examinations should be repeated regularly to monitor retention of these characteristics. Attention has to be paid to the morphology of the cells, the degree of differentiation (49-61), antigenic expression of tumor cells, possible infiltration by mouse stromal tissue and other secondary events (50, 61). Immunological studies include not only the analysis of tumor markers (human chorionic gonadotropin, carcinoembryonic antigen) (49, 60), but also the presence of tumor cell specific antigens (62) detected by monoclonal antibodies.

Biochemical characterization of tumor lines can be based upon general parameters such as DNA content, chromosome studies (56, 63), isoenzyme pattern (50, 60, 61, 64), or upon specific features of the tumor line such as the presence of hormone receptors (65), cellular enzyme profile (66) and serological detection of tumor markers.

Testing for chemotherapeutic sensitivity to particular drugs is a method frequently employed to characterize the tumor lines in relation to the patient's tumor tissue. Obviously, the sensitivity pattern should be retained if the tumor lines are to be used continuously for new drug development.

Tumor measurement and parameters

Subcutaneous xenografts offer a good visualization of tumor growth, and periodic tumor measurements can be carried out easily to follow volume progression or regression. Diameters are measured with slide calipers and results expressed as a measure of size (product of two perpendicular diameters) (39), tumor weight ($1/2 \times \text{length} \times \text{width}^2$) (7) or tumor volume ($1/6 \times \pi \times \text{mean tumor diameter}^3$ or $1/2 \times \text{length} \times \text{width} \times \text{height}$) (67-69).

At the time of progressive growth when tumors have reached a diameter of about 5 mm, chemotherapy experiments are started in randomized groups of 5-10 animals. Drugs are usually injected i.p., but in some instances the i.v. or s.c. route has been used. Drug doses and schedules vary considerably between institutes because of differences in maximum tolerated doses between mouse strains or because of different experimental approaches. For example, a treatment schedule may be chosen in which a single LD10 dose is employed (14), or lower doses may be administered repeatedly (67, 69). Especially in slow growing tumor lines, frequent injections are preferred in an attempt to mimic the clinical situation.

Maximum effects of drug treatment are usually obtained within 3 to 4 weeks of treatment. Regarding tumor size calculations, the effects are classified as remission (product of two diameters $\leq 50\%$ of initial value), minimal regression (51-75%), no change (76-124%) or progression ($\geq 125\%$) (39). If tumor weights are measured, the mean tumor weights

Table III. Direct comparison of drug effects in the nude mouse with clinical results in the donating patient's tumor

Tumor type	Number of patients	Number of treatment comparisons	Evaluation criteria - nude mouse	Mouse/patient ^a				Ref.
				R/R	P/P	R/P	P/R	
Colorectal ^b	4	4	SGD>2	1	3	-	-	59
lung SCLC ^b	6	8	SGD>2	5	2	1	-	70
lung NSCLC ^b	9	12	SGD>2	-	12	-	-	70
Colorectal	1	1	MTW T/C%	1	-	-	-	71
Breast	1	1	"	-	1	-	-	71
Gastric	2	3	"	-	3	-	-	71
Colorectal	14	22	PR+CR	2	19	-	1	39
Gastric	6	9	PR+CR	4	5	-	-	39
Lung NSCLC	4	6	PR+CR	6	-	-	-	39
Lung SCLC	2	4	"	-	4	-	-	72
Melanoma	4	4	"	-	3	1	-	39
Sarcoma STS	2	2	"	-	2	-	-	39
Penile	1	1	PR+CR	1	-	-	-	39
Wilms	1	2	PR+CR	2	-	-	-	39
Thyroid	1	1	PR+CR	1	-	-	-	39
Melanoma	17	37	GD>2	8	25	3	1	73
Total	77 ^a	117		31	79	5	2	

^a Comparison nude mouse/patient.^b Tumor lines in artificially immune-deprived mice.

R = Clinical response (CR+PR) or meets evaluation criteria in the nude mouse.

P = Clinical progression or does not meet evaluation criteria in the nude mouse.

are calculated for each day of measurement and are expressed as the mean tumor weight change (MTW) of treated (T) over control (C) tumors multiplied by 100, T/C % (7). An optimum T/C of < 20% is considered necessary to demonstrate moderate sensitivity to a drug. A reproducible T/C < 10% is considered significant sensitivity. Institutes employing tumor volume measurements may express their chemotherapy effects either in specific growth delay (14, 67, 68) or in tumor growth inhibition (69). The usual procedure used to measure growth delay is to calculate the time taken for the treated (TT) and control tumors (TC) to reach a chosen volume. The difference (TT - TC) equals the actual tumor growth delay. Because tumor types may differ widely in growth rate, the actual tumor growth delay is related to the tumor doubling time. The resulting specific growth delay could also be described as the number of volume doubling times by which growth is delayed. If results are expressed as tumor growth inhibition, at each evaluation day volumes are calculated in relation to the initial tumor volume. The optimal ratio of the mean relative tumor volume in treated mice (T) over that in control mice (C) multiplied by 100 will result in the maximal percentage growth inhibition (100% - T/C %) to be obtained by the drug. Activity criteria for both specific growth delay and tumor growth inhibition have not yet been standardized.

Predictive value for the individual patient

An essential requirement for the validation of human tumor lines in anticancer drug development is a positive correlation

of drug effects in the nude mouse with clinical results in the donating patient's tumor. Several comparative studies have been published although for a number of reasons these are difficult to perform: the patient may not have been treated with chemotherapy, may have received combination chemotherapy at a schedule difficult to apply in mice or may not have had a measurable tumor lesion to assess treatment response.

From literature reports on chemotherapy studies in human tumor xenografts grown s.c., we have found 117 direct comparisons between laboratory data and patients' treatment. The tumors originated from 77 patients (Table III). Treatment comparisons of refs. 59 and 70 (19 patients) were performed with transplants in artificially immune-deprived mice using specific growth delay as a measure of activity (14). For all other comparisons between the experimental model and the patient, experiments were carried out in the nude mouse. Antitumor effects were expressed as mean inhibition of tumor weight/volume, as partial/complete response or as growth delay (Table III).

In 31/33 treatment comparisons the mouse xenograft model predicted well for clinical response, and in 79/84 for clinical resistance to drug treatment. Unfortunately, most of these positive correlations are based upon results obtained in clinically unresponsive tumor types (*i.e.* melanoma, colorectal cancer). In view of the above, clinical oncologists would obviously prefer to select the optimal treatment for the individual patient in the laboratory. Multiple limitations of human tumor lines preclude such a routine approach. The major limitations have already been discussed: a low take

Table IV. Comparison of responsiveness to chemotherapy between human tumor lines and in the clinic (from ref. 14)

Tumor type	Number of lines	Average specific growth delay ^a	Clinical complete response rate (%)
Testicular cancer	2 - 3	5.7	70
Lung cancer SCLC	2 - 3	4.2	39
Mammary cancer	2 - 5	1.9	15
Melanoma	3 - 4	1.0	4
Colorectal cancer	8 - 10	0.8	3
Lung cancer NSCLC	3 - 5	0.5	5

a. Average SGD using the three single drugs in clinical use that are most effective in each tumor type.

rate of take of certain tumor types, the time delay due to serial transfer and the high costs involved.

Prediction of antitumor activity in a specific tumor type

Another possibility to validate the role of human tumor lines in anticancer drug development is to assess whether xenografts of a certain tumor type will select the clinically relevant drugs for that tumor type and reject drugs without activity.

In view of the variability in antitumor response to standard treatment between patients with the same tumor type, the use of a panel of lines of the same tumor type is mandatory when evaluating the effect of a drug. As a result of this diversity Bellet *et al* (74) proposed already in 1979 to use human tumor lines of at least 14 patients with the same tumor type as a secondary screen for new compounds: "It has been our clinical experience that no single patient's response to systemic chemotherapy will be the same as all patients having the same type of cancer... We believe that it is unreasonable to expect one patient's tumor to be representative of all patients' tumors of the same histologic variety". This opinion is supported by the chemotherapy responses reported from the MX-1 human tumor xenograft. Since 1975 the MX-1 xenograft has been employed in the NCI tumor panel in secondary screening, after the line had been established from a patient with a poorly differentiated infiltrating duct cell carcinoma of the breast. Evaluation of the sensitivity of the MX-1 as an s.c. implanted xenograft revealed that cisplatin was effective, whereas doxorubicin, 5-fluorouracil (5-FU) and methotrexate (MTX), all known for their clinical activity in advanced breast cancer, were ineffective (7).

In a review of data generated at his institute, Steel reported that human tumor lines roughly maintain the same chemosensitivity pattern as would be expected from clinical results (14). Using three single drugs clinically active in a specific tumor type, the average specific growth delay achieved in a series of lines of that tumor type correlated well with the clinical complete response rate (Table IV).

In order to assess the predictability of a panel of human tumor lines of a specific tumor type for clinically effective drugs, we shall analyze publications dealing with this subject.

Melanoma. Melanoma is a tumor type for which standard

chemotherapy is not available. Dacarbazine (DTIC) appears to be the best clinically active drug but response rates have been low (PR + CR less than 20%). Chemotherapy studies in human melanoma xenografts were first published in 1975 (75). More reports on the activity of drugs in melanoma lines have followed (68, 73, 76). Several drugs showed activity in a variable number of lines. The extent of preclinical activity observed can hardly be correlated to the clinical efficacy of these drugs (procarbazine, DTIC, MeCCNU, cisplatin, ifosfamide, mitomycin C, and doxorubicin).

Colorectal cancer. Colorectal cancer, like melanoma, is a tumor type in which no good standard treatment is known. 5-FU is the most effective drug with a clinical response rate of about 20%. This unresponsiveness is reflected by chemotherapy trials in the nude mouse (59, 60, 77-79). Surprisingly two analogs of doxorubicin were found to be promising agents in this tumor type in nude mice (77). Of these, deoxydoxorubicin was evaluated in clinical practice but did not show significant activity in colorectal cancer. Besides 5-FU, other drugs have also been designated by colon cancer lines, as active such as MeCCNU, BCNU, chlorozotocin, mitomycin C, cyclophosphamide. However, activity of a particular drug was often based on efficacy in one of a series of colon cancer lines.

Lung cancer. Chemotherapy experiments in a panel of human lung cancer lines are still limited.

SCLC: a number of drugs is known to be active in small cell lung cancer (SCLC), such as doxorubicin, etoposide, cyclophosphamide, cisplatin. Shorthouse *et al* showed that human tumor lines of this tumor type were sensitive to these drugs (80). In their experiments, procarbazine was also indicated to be active, which could not be confirmed in a phase II clinical trial in SCLC patients (81).

NSCLC: drugs with a low activity in non-small cell lung cancer (NSCLC) are cisplatin, vindesine and etoposide, but these cannot be considered standard chemotherapy. In five NSCLC lines cyclophosphamide and CCNU incidentally showed activity in one line each, while a number of other drugs were inactive (80). Doxorubicin had to be considered ineffective in two NSCLC lines studied by Giuliani *et al* (76).

Table V. Growth inhibition (%) * by single drugs in human ovarian cancer lines.

Tumor line	CDDP (%)	CTX (%)	DXR (%)	HMM (%)	MTX (%)	5FU (%)					
Ov. Pe	+	(35)	-	(36)	+	(60)	-	(30)	(19)		
MRI-H-207	++	(100)	++	(100)	++	(99)	++	(100)	(75)	(37)	
Ov. Me	+	(64)	++	(98)	++	(95)	++	(99)	+	(52)	(14)
Ov. He	+	(56)	-	(10)	+	(66)	+	(73)	-	(7)	(43)
GH	++	(77)	-	(44)	-	(87)	-	ND ^a	-	(14)	
S. Ri (C)	++	(92)	+	(62)	-	(35)	+	(61)	ND	-	(26)
ICo	-	(46)	-	(5)	-	(10)	-	(36)	ND	-	(0)
FKo	-	(14)	-	(4)	-	(8)	-	(35)	ND	-	(21)
FMa	++	(99)	+	(60)	++	(76)	++	(95)	-	(0)	(54)
A 2780S	+	(64)	++	(82)	++	(81)	++	(80)	ND	-	(4)

* Growth inhibition $\leq 50\%$ = -; $>50\%$ to $\leq 75\%$ = +; $> 75\%$ = ++.
 not done

Ovarian cancer. Standard single agents in ovarian cancer are cisplatin and alkylating drugs (cyclophosphamide, melphalan), while activity has also been shown for doxorubicin and hexamethylmelamine.

Chemotherapy studies in 10 human ovarian cancer lines differing in histological subtype and tumor doubling time clearly indicated a difference in sensitivity to each of six drugs (Table V) (82). This panel of 10 tumor lines appeared to select the clinically relevant drugs. The number of lines in which over 75% growth inhibition could be achieved was 3/10 for cyclophosphamide, 4/10 for doxorubicin, 4/10 for cisplatin and 5/10 for hexamethylmelamine. MTX and 5-FU lacked effects on the panel and are known to be irrelevant drugs for ovarian cancer in the clinical situation.

Head and neck cancer. Cisplatin, MTX, bleomycin and 5-FU are considered to be the most active single drugs in head and neck squamous cell cancer. Reports on chemotherapy experiments in tumor lines of this malignancy are limited. In a study of 14 lines cisplatin and bleomycin appeared to be the most active (83). However, no major growth delay was observed in 16 lines treated with MTX (84).

Breast cancer. In breast cancer a series of drugs are known to be active: doxorubicin, MTX, 5-FU, cyclophosphamide, mitomycin C. Breast cancer belongs to those malignancies that are difficult to establish as human tumor lines. The human tumor line MX-1 has been used by other investigators (85, 86) who confirmed its sensitivity pattern as has been reported earlier by Ovejera and Houchens (7). Unfortunately, this tumor line is not sensitive to doxorubicin, while cisplatin exerts major tumor growth inhibition. A number of breast cancer lines have been applied for chemotherapy testing. In the studies reported by Bailey et al (87), the best results were obtained in five lines (grown in artificially immune-suppressed mice) with melphalan, or a combination of CMF (CTX, MTX, 5-FU) or DV (DXR + VCR) (Table VI). Individual sensitivity to drugs in these lines varied greatly. Three other lines were found to be sensitive to

Table VI. Specific growth delay by chemotherapy in five breast cancer lines (From ref. 87).

Drug	Xenograft No.				
	99	100	104	105	106
Melphalan	2.7	3.1	2.2	1.9	2.2
DXR	4.1	3.1	0.5	1.2	0.6
CTX	3.2	1.7	1.1	1.5	0.9
5-FU	2.2	2.5	0.8	2.4	0.5
VCR	2.5	1.2	0.7	0.5	0.4
MTX	1.0	0.5	0.6	0.5	0.2
CTX+MTX+5-FU	4.9	3.3	1.5	2.8	1.5
DXR+VCR	6.5	3.8	0.8	1.5	1.0

doxorubicin (76). Except for MX-1, studies of agents inactive in breast cancer in clinical practice have yet to be carried out in xenografts of this tumor type.

Limitations of the xenograft model

Human tumor lines grown s.c. in nude mice have their disadvantages as compared to the use of murine tumor models. The major limitations are the high costs involved in the breeding and maintenance of the animals. Laboratory facilities should be specific-pathogen free, while the personnel has to be well trained to ensure high quality experiments. A practical problem is the extensive time needed to establish and characterize a human tumor line which precludes its use as a predictive model for the individual patient. Chemotherapy experiments may take two to three months which limits the number of drugs that can be studied.

Another problem in the establishment of tumor lines representing a particular human malignancy is the variation in transplantability. Theoretically a good panel of lines should contain all histological subtypes, various degrees of differentiation and different tumor doubling times. Tissue factors however, such as the degree of differentiation, the origin of the fragments (primary tumor, metastasis) or the

histological (sub)type, are known to be of influence on the successful growth as xenografts.

Tumor characteristics of established lines need to be monitored continuously. Changes in latency period and growth rate with a concurrent change in chemosensitivity have been described for two established melanoma xenografts by Fodstad *et al* (58). Changes in growth rate with a transformation of histological and biochemical characteristics have also been reported for a nephroblastoma line (61) and for a colon cancer, a lung cancer and a lymphoma line (88). To circumvent this problem, many laboratories freeze a large amount of tissue from early passages so that the original tumor line can be re-established in case of a dramatic change in tumor characteristics.

With regard to chemotherapy experiments in human tumor lines and in patients, several pharmacologic and pharmacokinetic differences should be taken into account:

a. Therapeutic studies in human tumor xenografts are started when tumors are small and in the log-phase of growth, while in patients treatment is begun when tumors are well established and relatively slow growing;

b. Drug doses, methods of drug administration, bioavailability, metabolism and elimination in mice and man may differ;

c. The assessment of the maximum tolerated dose in mice according to weight loss and death is different from the determination of dose-limiting side effects in patients;

d. The assessment of response to treatment in xenografts by comparison of the tumor volume (weight) in treated animals with that of control animals differs from response criteria in patients.

From the data on active conventional anticancer agents reviewed above, it appears that the predictability of tumor lines is better for responsive tumor types (mammary cancer, ovarian cancer) than for non-responsive tumors (colorectal cancer, melanoma).

It should be noted that false negative results from chemotherapy experiments with human tumor lines have been reported. This is clearly illustrated by the lack of significant activity of MTX in head and neck cancer xenografts (83, 84), whereas it is an active agent in this tumor type in clinical practice. For true positives, the degree of activity in the nude mouse cannot be directly related to clinical activity. Based on xenograft testing, for instance hexamethylmelamine would have been predicted to be more active than cisplatin or cyclophosphamide in ovarian cancer (82, 89).

Conclusions and future perspectives

The data obtained thus far indicate that human tumor lines in nude mice are realistic models of human cancer and as such may be useful tools in anticancer drug development. Unfortunately, due to a number of practical limitations such as low take rate and slow tumor growth, they cannot be applied routinely in the selection of the optimal chemotherapy for the individual patient. These limitations do not preclude the use

of human tumor lines in the nude mouse in a new drug development program for anticancer agents. Relatively fast-growing human tumor lines have already been used in the tumor panel of the NCI drug screening program. Recent studies have demonstrated that the successful application of xenografts will require the use of a representative panel of lines per tumor type. The ideal size of such panels has to be determined on the basis of a cost-benefit analysis and statistical considerations. The high cost level of xenograft testing will limit its use as a method of primary screening. A model which is to be used in primary screening aiming at the identification of new "lead compounds" should be a high-capacity, low-cost model. Recently, Fiebig proposed to increase the capacity of a xenograft-based secondary screening system by pre-screening of new agents *in vitro*, using a xenograft-derived clonogenic assay (90). A potential danger of this approach is that compounds requiring bioactivation *in vivo* may be missed when no further *in vivo* results are available.

For the near future, the major role of human tumor lines will probably be in advanced screening of promising agents identified by other test methods. An interesting initiative in this direction has recently been developed by the Early Clinical Trials Group of the European Organization for Research and Treatment of Cancer (EORTC). In a cooperative effort of several specialized European xenograft laboratories, the value of human tumor lines in the prediction of drug efficacy in phase II trials will be investigated, both retrospectively and prospectively. Experiments also include clinically inactive agents in order to obtain an estimate of the risk of false positives. The aim of phase II clinical trials is to determine the response rate per tumor type. If panels of tumor lines in the nude mouse appear to be predictive for the clinical response of a drug, this approach may definitely reduce the number of trials with negative results and rapidly indicate sensitive tumor types.

References

- Narayan VL: Strategy for the discovery and development of novel anticancer agents. In Structure-activity relationships of anti-tumour agents (Reinhoudt DN, Connors TA, Pinedo HM and Van der Poel KW, eds). The Hague/Boston/London, Martinus Nijhoff Publishers, 1983, pp 5-22.
- Driscoll JS: The preclinical new drug research program of the National Cancer Institute. *Cancer Treat Rep* 68: 63-76, 1984.
- Venditti JM, Wesley RA and Plowman J: Current NCI preclinical antitumor screening *in vivo*: Results of tumor panel screening 1976-1982 and future directions. In *Advances in pharmacology and chemotherapy* (Garattini S, Goldwin A, Hawkins S, eds). Orlando, Academic Press, 1984: 20, 1-20.
- Goldin A, Venditti JM, MacDonald JS, Muggia FM, Henney JE and DeVita Jr VT: Current results of the screening program at the division of cancer treatment, National Cancer Institute. *Eur J Cancer* 17: 129-142, 1981.
- Corbett TH, Griswold Jr DP, Roberts BJ, Peckham JC and Schabel Jr FM: Evaluation of single agents and combinations of chemotherapeutic agents in mouse colon carcinomas. *Cancer* 40: 2660-2680, 1977.

The material on this page was copied from the collection of the National Library of Medicine by a third party and may be protected by U.S. Copyright law

- drug fast in the recent on of vel of to be satis-fying model t the high-0 in-ning ng a inger on in are
- lines gents re in larly for era-bor-n of uth ude the s to s of the tely idly
- ivcl ooll rts. nal cal 76-ve-to, nd on 77. Jf u-7.
- 6 Martin DS, Fugmann RA, Stolfi RL and Hayworth PE: Solid tumor animal model therapeutically predictive for human breast cancer. *Cancer Chemother Rep* 5: Part 2, 89-109, 1975
- 7 Ovejero AA and Houchens DP: Human tumor xenografts in athymic nude mice as a preclinical screen for anticancer agents. *Scand Oncol* 8: 386-393, 1981
- 8 Staquet MJ, Byar DP, Green SB and Rozencweig M: Clinical prediction of transplantable tumor systems in the selection of new drugs for all tumors: Rationale for a three-stage strategy. *Cancer Treat Rep* 65: 783-785, 1983
- 9 O'Dwyer PJ, Eisenhauer EA and Hoth DF: Deoxygymnasterin in lymphoid malignancies. An overview. *Invest New Drugs* 5: 1987 in press
- 10 Eisenhauer E, Johnston JB, Barr R, Feldman L, Maksymuk A, Scott G, Sutton D, Verner P, Walde D and Zaczek D: 2'-Deoxygymnasterin (DCF) in hairy cell leukemia (HCL). *Invest New Drugs* 5: 1987 in press
- 11 Schabel RF, FM, Griswold Jr DP, Corbett TH, Russell Laster Jr W, Mayo JG and Lloyd HJ: Testing therapeutic hypotheses in mice and man: observations on the therapeutic hypotheses in mice and man: observations on the therapeutic activity against advanced solid tumors of mice treated with anticancer drugs that have demonstrated or potential clinical utility for treatment of advanced solid tumors of man. *In: Cancer drug development. Part B. Methods in cancer research* (Busch H, DeVita V eds). New York: Academic Press Inc, 1979, pp 3-51
- 12 Martin DS, Stolfi RL and Sawyer RC: Commentary on "Clinical prediction of transplantable tumor systems in the selection of new drugs for solid tumors: rationale for a three-stage strategy". *Cancer Treat Rep* 68: 1317-1318, 1984
- 13 Boyd MR, Shoemaker RH, Alley MC, Seudiro DA, Monks A, Fine DL, Mayo JG and Chabner BA: Drug development. In: Roth JA, Ruckdeschel JC, and Wessenberg TH (eds) W.B. Saunders (in press).
- 14 Steel GG, Courtney VD and Peckham MD: The response to chemotherapy of a variety of human tumour xenografts. *Br J Cancer* 47: 1-13, 1983
- 15 Isaacson JH and Cattanaeh BM: Report. *Mouse News Lett* 27: 31, 1962
- 16 Flanagan SP: "Nude", a new hairless gene with pleiotropic effects in the mouse. *Genet Res* 8: 295-309, 1966
- 17 Pantelouris EM: Absence of a thymus in a mouse mutant. *Nature* 217: 370-371, 1966
- 18 Harsen CT: The nude gene and its effects. *In: The nude mouse in experimental and clinical research* (Fogh J and Giovanella BC, eds). New York, Academic Press, 1978, pp 1-13
- 19 Rygaard J: Immuno deficiency in nude mice. *In: Thymusaplastic nude mice and rats in clinical oncology* (Bastien GBA et al, eds). Stuttgart, Gustav Fischer, 1981, pp 3-14
- 20 Fujiwara K, Tamura T, Taguchi P, Hirano N and Ueda K: Wasting disease in nude mice infected with facultatively virulent mouse hepatitis virus II. *In: Proc Sec Int Workshop on nude mice*. Univ. of Tokyo Press, Tokyo/Gustav Fischer Verlag, Stuttgart, 1977, pp 53-60
- 21 Ueda K, Tamura T, Machi K and Fujiwara K: An outbreak of Sendai virus infection in a nude mouse colony. *In: Proc Sec Int Workshop on nude mice*. Univ. of Tokyo Press. Tokyo Gustav Fischer Verlag, Stuttgart, 1977, pp 61-69
- 22 Rygaard J and Povlsen CO: Heterotransplantation of a human malignant tumor to "nude" mice. *Acta Pathol Microbiol Scand* 77: 758-760, 1969
- 23 Giovanella BC, Stehlin JS, Williams LJ, Lee SS and Shepard RC: Transplantation of human cancers into nude mice. *Cancer* 42: 2269-2281, 1978
- 24 Azar HA, Fernandez SB, Broff LM and Speeter SC: Human tumor xenografts in nude mice: current experience and chemotherapy trials. *Pathol Ann* 19, part 2: 245-268, 1984
- 25 Hanna N, Davis TW and Fidler IJ: Environmental and genetic factors determining the level of NK activity of nude mice and affect their suitability as models for experimental metastases. *Int J Cancer* 30: 371-376, 1982
- 26 Sharp AK and Colston MJ: The regulation of macrophage activity in congenitally athymic mice. *Eur J Immunol* 14: 102-105, 1984
- 27 Nauta MM, Böven E, Schijper HMM, Erkelens CAM and Pinedo HM: Enhanced transplantability of human ovarian cancer lines in cyclophosphamide-pretreated nude mice. *Br J Cancer* 54: 331-335, 1986
- 28 Committee on care and use of the "nude" mouse: Guide for the care and use of the nude (thymus-deficient) mouse in biomedical research. *ILAR News* 19, M1-M20, 1978
- 29 Bogden AE, Hassell PM, LePage DJ, Kellon DE, Cobb WR and Escher HJ: Growth of human tumor xenografts implanted under the renal capsule of normal immunocompetent mice. *Exp Cell Biol* 47: 281-293, 1979
- 30 Bogden AE, Cobb WR, LePage DJ, Hassell PM, Guirkin TA, Ward A, Kellon DE and Escher HJ: Chemotherapy responsiveness of human tumors as first transplant generation xenografts in the normal mouse, six-day subrenal capsule assay. *Cancer* 48: 10-20, 1981
- 31 Griffin TW, Bogden AE, Reich SD, Antonelli D, Hunter RE, Ward A, Yu DT, Greene HL and Costanza ME: Initial clinical trials of the subrenal capsule assay as a predictor of tumor response to chemotherapy. *Cancer* 52: 2185-2192, 1983
- 32 Dumont P, Van der Elst EP, Jabri M, Lejeune F and Atassi G: Chemosensitivity of human melanoma xenografts in immunocompetent mice and its histological evaluation. *Int J Cancer* 33: 447-451, 1984
- 33 Aamdal S, Fodstad O, Kaalhus O and Pihl A: Chemosensitivity profiles of human cancers assessed by the 6-day SRC assay on serially xenografted tumors. *Int J Cancer* 37: 579-587, 1986
- 34 Giovanella BC, Stehlin JS Jr, Shepard RC and Williams LJ: Correlation between response to chemotherapy of human tumors in patients and in nude mice. *Cancer* 52: 1146-1153, 1983
- 35 Edelstein MD, Fiebig HH, Smink T, Van Putten LM and Schuchardt C: Comparison between macroscopic and microscopic evaluation of tumor responsiveness using the subrenal capsule assay. *Eur J Cancer Clin Oncol* 19: 995-1009, 1983
- 36 Edelstein MR, Smink T, Ruiter DJ, Visser W and Van Putten LM: Improvements and limitations of the subrenal capsule assay for determining tumor sensitivity to cytostatic drugs. *Eur J Cancer Clin Oncol* 12: 1549-1556, 1984
- 37 Bennett JA, Pilon VA and MacDowell RT: Evaluation of growth and histology of human tumor xenografts implanted under the renal capsule of immunocompetent and immunodeficient mice. *Cancer Res* 45: 4963-4969, 1985
- 38 Suzuki K and Sudo K: Heterotransplantation of human stomach carcinoma in nude mice. *Gann* 70: 109-114, 1979
- 39 Fiebig HH, Schuchardt C, Henss H, Fiedler L and Lohr GW: Comparison of tumor response in nude mice and in the patients. *Behring Inst Mitt* 74: 343-352, 1984
- 40 Nakatani K, Takahashi S, Shiratori T and Konishi Y: Heterotransplantation of human gastric carcinoma into nude mice. *World J Surg* 3: 641-650, 1979
- 41 Shimamoto Y, Kameya T, Nagai K and Hirohashi S: Transplantation of human tumors in nude mice. *J Natl Cancer Inst* 56: 1251-1260, 1976
- 42 Fogh J, Orfeo T, Tiso J, Sharkey FE: Twenty-three new human tumor lines established in nude mice. *Exp Cell Biol* 46: 229-239, 1980
- 43 Sharkey FE, Fogh JM, Haydu SJ, Fitzgerald PJ and Fogh J: Experience in surgical pathology with human tumor growth in the nude mouse. *In: The nude mouse in experimental and clinical research* (Fogh J and Giovanella BC, eds). New York, Academic Press, 1978, pp 187-214
- 44 Povlsen CO and Rygaard J: Heterotransplantation of human adenocarcinomas of the colon and rectum to the mouse mutant nude. A study of nine consecutive transplantations. *Acta Pathol Microbiol Scand* 79: Section A 159-169, 1971
- 45 Povlsen CO: Heterotransplantation of human malignant melanomas

- to the mouse mutant nude. *Acta Pathol Microbiol Scand* 84: Section A 9-16, 1976.
46. Mattern J, Ways K, Haag D, Toomes H and Volm M: Different growth rates of lung tumours in man and their xenografts in nude mice. *Eur J Cancer* 16: 289-291, 1980.
47. Gazdar AF, Carney DN, Sims HL and Simmons A: Heterotransplantation of small-cell carcinoma of the lung into nude mice: comparison of intracranial and subcutaneous routes. *Int J Cancer* 38: 777-783, 1981.
48. Braakhus BJM, Smeekwerp G and Snow GB: The potential of the nude mouse xenograft model for the study of head and neck cancer. *Arch Otorhinolaryngol* 239: 69-79, 1984.
49. Yeager H, Bauml R, Pawin G and Phillips MJ: Relationship of histology of Wilms' tumor to growth characteristics of nude mice heterotransplants. *Cancer Res* 45: 2340-2349, 1985.
50. Neely JE, Ballard ET, Britt AL and Workman L: Characteristics of R5 pediatric tumors heterotransplanted into nude mice. *Exptl Cell Biol* 51: 217-227, 1983.
51. Teufel G, Kleine W, Günther M, Schwör D and Pfeiderer A: Growth of human ovarian carcinomas in thymusplastic nu/nu mice. In: *Thymusplastic nude mice and rats in clinical oncology* (Bastert GBA et al, eds). Stuttgart, Gustav Fischer Verlag, 1981, pp 111-116.
52. Boven E: Biology of human ovarian cancer xenografts. In: *Human tumor xenografts in anticancer drug development* (Winograd B, Peckham MJ and Pinedo HM, eds). Heidelberg, Springer Verlag, 1987 in press.
53. Friedlander ML, Russell P, Taylor JW and Tattersall MHN: Ovarian tumor xenografts in the study of the biology of human epithelial ovarian cancer. *Br J Cancer* 51: 319-333, 1985.
54. Kullander S, Rausing A and Tröpé C: Human ovarian tumours heterotransplanted to «nude» mice. *Acta Obstet Gynecol Scand* 57: 149-159, 1978.
- 54a. Hajdu SI, Lemos LB, Kozakewich H, Helson L and Beattie EJ: Growth pattern and differentiation of human soft tissue sarcomas in nude mice. *Cancer* 47: 90-98, 1981.
55. Gershwin ME, Ikeda RM, Kawakami TG and Owens RB: Immunobiology of heterotransplanted human tumors in nude mice. *J Natl Cancer Inst* 58: 1455-1461, 1977.
56. Wennerberg J: Changes in growth pattern of human squamous-cell carcinomas of the head and neck during serial passages in nude mice. *Int J Cancer* 33: 245-250, 1984.
57. Bailey MJ, Ormerod MG, Imrie SF, Humphreys J, Roberts JDB, Gazet JC and Neville AM: Comparative functional histopathology of human breast carcinoma xenografts. *Br J Cancer* 43: 125-134, 1981.
58. Fodstad O, Rolstad EK, Tveit KM and Pihl A: Spontaneous alteration in the growth rates of two human melanoma xenografts. Concurrent changes in chemosensitivity. *Eur J Cancer Clin Oncol* 19: 1175-1178, 1983.
59. Nowak K, Peckham MJ and Steel GG: Variation in response of xenografts of colorectal carcinoma to chemotherapy. *Br J Cancer* 37: 376-384, 1978.
60. Fiebig HH, Widmer KH, Fiedler L, Wittekind O and Lohr GW: Development and characterization of 51 human tumor models for large bowel, stomach and esophageal cancers. *Dis Surg* 1: 225-235, 1984.
61. Rousseau-Mercé MF, Cottreau D and Kahn A: Isozyme pattern in serially transplanted childhood tumors. *Cancer Res* 44: 1163-1166, 1984.
62. Thomson DMP, Neville AM, Phelan K, Schwartz R, Scanzano and Vandocrode JP: Human cancers transplanted in nude mice retain the expression of their organ specific neonatogens. *Eur J Cancer Clin Oncol* 17: 1191-1197, 1981.
63. Ishii S, Yamawaki S, Sai T, Usui M, Ubayama Y, Minami A, Yagi T, Izu K and Kohayashi M: Characteristics of human soft tissue sarcomas in xenografts and *in vitro*. *Clin Orthop* 173: 251-261, 1983.
64. Nakamura T and Kitagawa T: Anticancer drug screening test with LDH in nude mouse bearing bone and soft part sarcoma. *Cancer* 56: 1112-1116, 1985.
65. Brunner N, Bester GB, Poulsen HS, Spang-Thomsen M, Engelholm SA, Vindlov L, Nielsen A, Tommstrup N and Elling F: Characterization of the T61 human breast carcinoma established in nude mice. *Eur J Cancer Clin Oncol* 21: 833-843, 1985.
66. Grant AG, Duke D and Hernon-Taylor J: Establishment and characterization of primary human pancreatic carcinoma in continuous cell culture and in nude mice. *Br J Cancer* 49: 149-151, 1979.
67. Shorthouse AJ, Peckham MJ, Smyth JF and Steel GG: The therapeutic response of bronchial carcinoma xenografts: a direct patient-xenograft comparison. *Br J Cancer* 47: suppl IV: 142-145, 1980.
68. Fodstad O, Aast N and Pihl A: Response to chemotherapy of human malignant melanoma xenografts in athymic nude mice. *Int J Cancer* 35: 453-458, 1980.
69. Boven E, Nautia MM, Schlöper HMM, Elferink F, Van der Vijgh WJF and Pinedo HM: Secondary screening of platinum compounds in human ovarian cancer xenografts in nude mice. *Eur J Cancer Clin Oncol* 21: 1253-1260, 1985.
70. Shorthouse AJ, Smyth JF, Steel GG, Ellison M, Mills J and Peckham MJ: The human tumor xenograft - a valid model in experimental chemotherapy? *Br J Surg* 67: 715-723, 1980.
71. Fujita M, Hayata S and Taguchi T: Relationship of chemotherapy on human cancer xenografts in nude mice to clinical response in donor patient. *J Surg Oncol* 15: 211-219, 1981.
72. Fiebig HH, Neumann HA, Henss H, Koch H, Kaiser D and Arnold H: Development of three human small cell cancer models in nude mice. *Recent Results Cancer Res* 97: 77-86, 1985.
73. Osieka R: Studies on drug resistance in a human melanoma xenograft system. *Cancer Treat Rev* 11 Suppl A: 85-97, 1984.
74. Bellet RE, Danna V, Mastrangelo MKJ and Borden D: Evaluation of a «nude» mouse-human tumor panel as a predictive secondary screen for cancer chemotherapeutic agents. *J Natl Cancer Inst* 63: 1185-1188, 1975.
75. Povlsen CO and Jacobsen GK: Chemotherapy of a human malignant melanoma transplanted in the nude mouse. *Cancer Res* 33: 2790-2796, 1975.
76. Giuliani FC, Zirvi KA and Kaplan NO: Therapeutic response of human tumor xenografts in athymic mice to doxorubicin. *Cancer Res* 41: 325-333, 1981.
77. Giuliani FC, Zirvi KA, Kaplan NO and Goldin A: Chemotherapy of human colorectal tumor xenografts in athymic mice with clinically active drugs: 5-fluorouracil and 1-3-bis-(2-chloroethyl)-1-nitrosourea (BCNU), comparison with doxorubicin derivatives d'-deoxydoxorubicin and 4'-0-methyldoxorubicin. *Int J Cancer* 27: 5-13, 1981.
78. Osieka R, Houchens DP, Goldin A and Johnston RK: Chemotherapy of human colon cancer xenografts in athymic nude mice. *Cancer* 40: 2640-2650, 1977.
79. Houghton PJ and Houghton JA: Evaluation of single agent therapy in human colorectal tumor xenografts. *Br J Cancer* 37: 833-840, 1978.
80. Shorthouse AJ, Jones JM, Steel GG and Peckham MJ: Experimental combination and single-agent chemotherapy in human lung-tumour xenografts. *Br J Cancer* 46: 35-44, 1982.
81. Pedersen AG, Sörensen S, Aabo K, Dombrowsky P and Hansen HH: Phase II study of procarbazine in small cell carcinoma of the lung. *Cancer Treat Rep* 66: 273-275, 1982.
82. Boven E: Conventional agents in human ovarian cancer xenografts. In: *Human tumor xenografts in anticancer drug development* (Winograd B, Peckham MJ and Pinedo HM, eds). Heidelberg, Springer Verlag, 1987, in press.
83. Braakhus BJM, Schoevers EJ, Heinen ECM, Smeekwerp G and Snow GB: Chemotherapy of human head and neck cancer xenografts with three clinically active drugs: cisplatin, bleomycin and methotrexate. *Br J Cancer* 48: 711-716, 1983.
84. Braakhus BJM, Leyva A, Schoevers EJ, Boerigter GM, Schoragel JH and Snow GB: Lack of effect of methotrexate on known head and neck tumours transplanted in athymic nude mice. *Acta Otolaryngol (Stockh)* 99: 208-213, 1985.

- 85 Inoue K, Fujimoto S and Ogawa M. Antitumor activities of seventeen alkylating agents against human mammary carcinoma (MX-1) in nude mice. *Nagoya J Med Sci* 43: 89-100, 1981.
- 86 Inoue K, Fujimoto S and Ogawa M. Antitumor activities of seventeen alkylating agents against human mammary carcinoma (MX-1) in nude mice. *Cancer Chemother Pharmacol* 10: 182-186, 1983.
- 87 Bailey MJ, Gazet JC, Smith IE and Steel GG. Chemotherapy of human breast-carcinoma xenografts. *Br J Cancer* 42: 530-536, 1980.
- 88 Giehlholm SA, Brünner N, Spang-Thomsen M and Vindelev L. Genetic instability of human solid tumors grown in nude mice. In: *Immune-deficient animals* (Sordat B, ed) Basel, Karger 1984, pp 339-344.
- 89 Boven E, Nauta MM, Schläpfer HMM, Erkelens CAM and Pinedo HM. Superior efficacy of trimetazolin to hexamethylmelamine in human ovarian cancer xenografts. *Cancer Chemother Pharmacol* 18: 124-128, 1986.
- 90 Fiebig HH. Feasibility and usefulness of human tumor xenografts in secondary screening. *Invest New Drugs*, in press 1987.

Received January 12, 1987
Accepted January 29, 1987

Journal of General Virology, Vol 65, 843-847, Copyright © 1984 by
Society for General Microbiology

ARTICLES

Distinctive characteristics of crude interferon from virus-infected guinea-pig embryo fibroblasts

TR Winship, CK Fong and GD Hsiung

Crude interferon preparations from primary guinea-pig embryo cells infected with vesicular stomatitis virus strain T1026R1 were shown to be more sensitive to heat (37 degrees C), pH 2.0, and SDS than crude mouse interferon.

Since the proportion of antiviral activity lost after each treatment was nearly the same, the existence of a single fraction of antiviral activity sensitive to all three treatments was suggested. Support for this possibility was given by the finding that subjecting this guinea-pig interferon to any one of the treatments rendered it insensitive to the effects of the other two.

This Article

- ▶ [Alert me when this article is cited](#)
- ▶ [Alert me if a correction is posted](#)

Services

- ▶ [Email this article to a friend](#)
- ▶ [Similar articles in this journal](#)
- ▶ [Similar articles in PubMed](#)
- ▶ [Alert me to new issues of the journal](#)
- ▶ [Download to citation manager](#)

PubMed

- ▶ [PubMed Citation](#)
- ▶ [Articles by Winship, T. R.](#)
- ▶ [Articles by Hsiung, G. D.](#)

Agricola

- ▶ [Articles by Winship, T. R.](#)
- ▶ [Articles by Hsiung, G. D.](#)

The opinion in support of the decision being entered today was not written for publication and is not binding precedent of the Board.

UNITED STATES PATENT AND TRADEMARK OFFICE

**BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES**

Ex parte NANA K. AYISI

Appeal No. 2006-1608
Application No. 09/978,593

ON BRIEF

Before SCHEINER, MILLS, and GREEN, Administrative Patent Judges.

GREEN, Administrative Patent Judge.

DECISION ON APPEAL

This is a decision on appeal under 35 U.S.C. § 134 from the examiner's final rejection of claims 20, 22, 31 and 32, which read as follows:

20. The method according to claim 31, wherein the virus is human immunodeficiency virus (HIV), herpes simplex virus (HSV), human cytomegalovirus (HCMV), poliovirus (PV), measles virus (MV) or yellow fever virus (YFV).
22. The method according to claim 20, wherein the virus is HIV-1, HCMV, HSV-1 or HSV-2.
31. A method comprising: contacting a virus-infected cell with an extract from Ocimum gratissimum in an amount effective to inhibit cytopathic effects of the virus in the cell.
32. The method according to claim 20, wherein the virus is HIV.

Claims 20, 22, 31 and 32 stand rejected under 35 U.S.C. § 112, first paragraph, on the grounds that the specification fails to provide an enabling disclosure. In addition, claims 20 and 31 stand rejected under 35 U.S.C. § 102(b) as being anticipated by El-Said¹ as evidenced by Merck.² After careful review of the record and consideration of the issues before us, we reverse both rejections.

DISCUSSION

Claims 20, 22, 31 and 32 stand rejected under 35 U.S.C. § 112, first paragraph, "because the specification, while being enabling for inhibiting HIV viral replication in Vero cells and Molt4 clone 8 cells with an extract of O. gratissimum, does not reasonably provide enablement for the O. gratissimum extract to inhibit HIV viral replication in a mammal or any other cell line. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to practice the invention commensurate in scope with these claims." Examiner's Answer, page 3.

¹ El-Said et al. (El-Said), "An investigation into the efficacy of Ocimum gratissimum as used in Nigerian native medicine," Planta Medicine, pages 195-200 (1969).

² Merck Manual (Merck), Beers et al., editors, published by Merck Research Laboratories, Whitehouse Station, NJ, pp. 1293-1296, 1303-1306, 1312-1323, 2320-2324 and 2341-2343 (1999).

"[A] specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as in compliance with the enabling requirement of the first paragraph of § 112 unless there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support." In re Marzocchi, 439 F.2d 220, 223, 169 USPQ 367, 369 (CCPA 1971) (emphasis in original). "[It] is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain why it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement." Id. at 224, 169 USPQ at 370. Here, the examiner has not provided "acceptable evidence or reasoning which is inconsistent" with the specification, and therefore has not met the initial burden of showing nonenablement.

While the examiner engages in a Wands analysis, see In re Wands, 858 F.2d 731, 737, 8 USPQ2d 1400, 1403 (Fed. Cir. 1988) (noting that facts that should be considered in determining whether a specification is enabling include: (1) the quantity of experimentation necessary to practice the invention, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims), the examiner's primary concern appears to

be that "the use of in vitro tests is not an acceptable predictor of in vivo activity when claiming treatments to HIV." Examiner's Answer, page 6.

According to the examiner, the "[c]haracteristics of a compound's activity in vitro using purified or partially purified components generally differs significantly with the compound when used in a living body." Id. at 3. The examiner asserts that clinical correlation of in vitro activity to in vivo efficacy is generally lacking, as cultured cell lines "differ significantly from in vivo animal models." Id. at 4.

Moreover, as explained by the examiner, "[t]he greatly increased complexity of the in vivo environment as compared to the very narrowly defined and controlled conditions of an in vitro assay does not permit a single extrapolation of in vitro assays to human diagnostic efficacy with any reasonable degree of predictability." Id. The examiner cites Planchon,³ Kerr⁴ and Chomienne⁵ to demonstrate the lack of correlation of in vitro testing to in vivo efficacy. See id. at 5. The examiner then cites a statement by Joanne Shellenbach, a spokeswoman for the American Cancer Society, quoted in the

³ Planchon et al. (Planchon), "Differential Effects of Butyrate Derivatives on Human Breast Cancer Cells Grown as Organotypic Nodules in Vitro and as Xenografts in Vivo," In Vivo, Vol. 6, pp. 605-10 (1992).

⁴ Kerr et al. (Kerr), "The relationship between Cytotoxic Drug Exposure and Tumour Cell Kill, in Vitro and in Vivo," In Vivo, Vol. 5, pp. 385-88 (1991).

⁵ Chomienne et al. (Chomienne), "Discrepancy Between in Vitro and in Vivo Passaged U-937 Human Leukemic Cells: Tumorigenicity and Sensitivity to Differentiating Drugs," In Vivo, Vol. 2, pp. 281-88 (1988).

Washington Times,⁶ stating that results in animal models “cannot always be easily replicated in humans.” Id.

The examiner next cites Kirs⁷ for its teaching that “[t]he effect of an inhibitor is also dependent on the virus, inhibitor concentration and cell line used,” indicating that an “inhibitor may be effective in one cell line but not in another cell line for the same virus.” Id. at 6. Finally, the examiner cites Mitsuya⁸ and Sandstöm⁹ as evidence that a drug that showed promise as a treatment of HIV in vitro, suramin, was not correlated to in vivo efficacy. See id.

The invention that must be enabled to satisfy § 112 is the invention defined by the claims. See CFMT, Inc. v. Yieldup Int’l Corp., 349 F.3d 1333, 1338, 68 USPQ2d 1940, 1944 (Fed. Cir. 2003) (“Title 35 does not require that a patent disclosure enable one of ordinary skill in the art to make and use a perfected, commercially viable embodiment absent a claim limitation to that effect.”). Thus, when the claims are not limited to a method that achieves therapeutic or clinical efficacy, such efficacy is not required for the claims to be enabled.

⁶ Joyce Howard Price, Researchers test ‘smart-bomb’ cancer therapy, Washington Times, November 16, 2001, at 3.

⁷ Kirs⁷ et al. (Kirs⁷), “Broad-Spectrum Antiviral Activity of 2-β-D-Ribofuranosylselenazole-4-Carboxamide, a New Antiviral Agent,” Antimicrobial Agents and Chemotherapy, Vol. 24, No. 3, pp. 353-61 (1983).

⁸ Mitsuya et al. (Mitsuya), “Suramin Protection of T Cells in Vitro Against Infectivity and Cytopathic Effect of HTLV-III,” Science, Vol. 226, pp. 172-74 (1984).

⁹ Sandstöm et al. (Sandstöm), “Antiviral Therapy in AIDS Clinical Pharmacological Properties and Therapeutic Experience to Date,” Drugs, Vol. 34, pp. 372-90 (1987).

Here, the claims are directed to a "method comprising [] contacting a virus-infected cell with an extract from Ocimum gratissimum in an amount effective to inhibit cytopathic effects of the virus in the cell" (claim 31). Thus, while it is fair to say that the claims encompass a method that achieves a clinically effective therapeutic response, they do not require it. Cf. In re Cortright, 165 F.3d 1353, 49 USPQ2d 1464 (Fed. Cir. 1999) (claims to a method of "treating scalp baldness" could be enabled even if the method did not produce a full head of hair).

We conclude that the potential problems identified by the examiner may indeed complicate treatment of a HIV in a patient, but such problems need not be overcome in order to "contact[] a virus-infected cell with an extract from Ocimum gratissimum in an amount effective to inhibit cytopathic effects of the virus in the cell" - all that is required by the claims. Thus, the examiner has not adequately explained why practicing the claimed method would have required undue experimentation.

Moreover, a claim may encompass inoperative embodiments and still meet the enablement requirement of 35 U.S.C. § 112, first paragraph. See Atlas Powder Co. v. E.I. Du Pont De Nemours & Co., 750 F.2d 1569, 1576, 224 USPQ 409, 413 (Fed. Cir. 1984), In re Angstadt, 537 F.2d 498, 504, 190 USPQ 214, 218 (CCPA 1976), In re Cook, 439 F.2d 730, 732, 169 USPQ 298, 300 (CCPA 1971). And the stage at which an invention in this field become useful is well before it is ready to be administered to humans." In re Brana, 51 F.3d 1560, 1568, 34 USPQ2d 1436, 1442 (Fed. Cir. 1995). (While the Brana court referred

to “usefulness”, the rejection on appeal was for nonenablement. See id. at 1564, 34 USPQ2d at 1439.)

Therefore, as the examiner has failed to set forth a prima facie case of unpatentability under 35 U.S.C. § 112, first paragraph, we are compelled to reverse the rejection.

Claims 20 and 31 stand rejected under 35 U.S.C. § 102(b) as being anticipated by El-Said.

According to the rejection,

El-Said [] disclose[s] that an aqueous extract of O. gratissimum has been used in Nigerian herbal medicine for the treatment of fevers (see abstract). Fever is a symptom that is associated with viral or bacterial infections (as evidenced by . . . Merck . . .). Thus, the treatment of viral infection using an extract of O. gratissimum is anticipated by El-Said [].

Examiner’s Answer, page 7.

The burden is on the examiner to set forth a prima facie case of unpatentability. See In re Alton, 76 F.3d 1168, 1175, 37 USPQ2d 1578, 1581 (Fed. Cir. 1996). In order for a prior art reference to serve as an anticipatory reference, it must disclose every limitation of the claimed invention, either explicitly or inherently. See In re Schreiber, 128 F.3d 1473, 1477, 44 USPQ2d 1429, 1432 (Fed. Cir. 1997).

Appellant argues that El-Said “disclose[s] the chemotaxonomy and antibacterial testing of Ocimum gratissimum specimens.” Appeal Brief, page 11 (emphasis in original). Therefore, according to appellant, “[t]he invention, as claimed, is not anticipated by [El-Said] because the reference does not disclose

anti-viral testing and/or a method of use of Ocimum gratissimum for inhibiting the cytopathic effects of a virus-infected cell.” Id. at 12. We agree, and the rejection is reversed.

CONCLUSION

Because the examiner has failed to set forth a prima facie case of unpatentability, both rejections of record are reversed.

REVERSED

Toni R. Scheiner)	
Administrative Patent Judge)	
)	
)	
)	BOARD OF PATENT
Demetra J. Mills)	
Administrative Patent Judge)	APPEALS AND
)	
)	INTERFERENCES
)	
Lora M. Green)	
Administrative Patent Judge)	

George A. Loud, Esquire
BACON & THOMAS
Fourth Floor
625 Slaters Lane
Alexandria VA 22314-1176

The opinion in support of the decision being entered today was not written for publication and is not binding precedent of the Board.

UNITED STATES PATENT AND TRADEMARK OFFICE

**BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES**

Ex parte RAYMOND H. BOUTIN

Appeal No. 2006-1879
Application No. 10/010,114

ON BRIEF

Before SCHEINER, GRIMES, and LEOVITZ, Administrative Patent Judges.

GRIMES, Administrative Patent Judge.

DECISION ON APPEAL

This appeal involves claims to a method of transferring nucleic acids into cells, which the examiner has rejected as nonenabled. We have jurisdiction under 35 U.S.C. § 134. Because we conclude that enabling the claimed method does not require providing therapeutically effective gene therapy, we reverse.

Background

Methods for delivering nucleic acids to cells in vivo face several problems: “persistence in the biophase of the organism for a sufficient time to reach the target cell; recognition of the target cell and means for mediating transport of the genetic material through the cell membrane and into the cytoplasm of the cell; avoidance of degradation

within the cell by the reticuloendothelial system; and transport to and through the nuclear membrane into the nucleus of the cell where transcription of the genetic material can take place." Specification, page 2, lines 5-14. The specification discloses a "multifunctional molecular complex for the transfer of a nucleic acid composition to a target cell comprising . . . : 1) said nucleic acid composition; 2) one or more cationic polyamine components . . . ; [and] 3) one or more endosome membrane disruption promoting components." Page 12, lines 2-9.

"The core, or backbone[,] of the transfer moiety is the cationic polyamine, containing between 3 and 12 amines." Page 23, lines 17-18. The function of the cationic polyamine is "to overcome the incompatibility arising from the hydrophilic nature of the nucleic acid molecule and the lipophilic nature of the cell membrane." Id., lines 20-23.

"The next component of the transfer moiety is the endosome membrane disruption promoting component. . . . This can either comprise one or more lipophilic long chain alkyl groups attached through one or more of the nitrogen atoms of said polyamine, or can comprise a bridging group . . . through which there is attached a fusogenic peptide, or cholic acid or cholesteryl or derivative compound." Page 25, lines 28-37. This component "prevent[s] degradation of the nucleic acid molecule in a lysosome," page 16, lines 27-28, by "permit[ting] the complex to escape from the endosome, whereupon it can migrate into the nucleus of the target cell, and release the nucleic acid composition, whose genetic information can then be transcribed within said nucleus." Page 34, lines 2-6.

Discussion

1. Claims

Claims 1, 2, 5-9, and 17-52 are on appeal. Claims 3 and 4 are also pending; claim 4 has been objected to but not rejected, and claim 3 has been withdrawn from consideration by the examiner.

Claim 1 is representative and reads as follows:

1. A method for the transfer of a nucleic acid composition to cells, comprising the step of introducing a multifunctional molecular complex into cells,

wherein said multifunctional molecular complex comprises:

A) a nucleic acid composition; and
B) a transfer moiety comprising

- (i) one or more cationic polyamine components, wherein each cationic polyamine is non-covalently bound to said nucleic acid composition and comprises from three to twelve nitrogen atoms; and
- (ii) one or more endosome membrane disruption promoting components independently selected from (a) at least one lipophilic long chain alkyl group or (b) a fusogenic peptide, cholic acid or cholesterol group or a derivative thereof;

wherein said multifunctional molecular complex transfers said nucleic acid composition to said cells.

Thus, claim 1 is directed to a "method for the transfer of a nucleic acid composition to cells." The claim is not limited to cells in culture or in a subject, so the claim encompasses both in vitro and in vivo methods. The claim comprises "introducing . . . into cells" a multifunctional complex comprising a nucleic acid composition; a cationic polyamine comprising three to twelve nitrogen atoms, noncovalently bound to the nucleic acid composition; and an endosome disrupting agent (which can be a

lipophilic long chain alkyl group, a fusogenic peptide, cholic acid, a cholesteryl group, or a derivative) attached to a nitrogen of the polyamine component via specified linkages.

2. Enablement

The examiner rejected claims 1, 2, 5-9, and 17-52 under 35 U.S.C. § 112, first paragraph, for nonenablement. The examiner focused on the aspect of the claimed method that involves transferring a nucleic acid encoding a therapeutic protein into cells.¹ The examiner concluded that the specification is enabling for a method of transferring a nucleic acid encoding a therapeutic protein into cells in vitro but is not enabling for the same method carried out in vivo. See the Examiner's Answer, page 3.

The examiner reasoned that "[t]he in vivo aspect of claims 1, 2, 5-9 and 17-52 is interpreted as gene therapy as the specification does not disclose a use for delivering a therapeutic protein other than for therapeutic purposes." Id. The examiner noted that the instant application has an effective filing date of September 28, 1994,² and cited several references as evidence that undue experimentation would have been required to successfully carry out gene therapy as of that date. Id., pages 4-6.

The examiner noted that the specification does not "disclose any particular DNA sequences that can be administered by applicant's claimed methods" to treat any specific disease. Id., page 6. The examiner summarized the most relevant working examples:

¹ The examiner restricted the claims based on the type of protein encoded by the transferred nucleic acid. See the restriction requirement mailed August 13, 2003. Appellant elected the claims directed to a method of transferring a nucleic acid encoding a therapeutic agent. See the paper filed September 11, 2003. The examiner has stated that "[b]ased on this election . . . claims 1, 2[,] 4-9, [and] 17-52, are interpreted as methods of delivering a therapeutic agent using applicant's novel multifunctional molecular complex." Examiner's Answer, pages 7-8.

² The instant application claims benefit under 35 U.S.C. § 120 of the filing date of application serial number 08/314,060, filed September 28, 1994.

Example 11 teaches the expression of lacZ when a plasmid comprising a β -galactosidase gene complexed to a transfer moiety of the invention is injected into mouse thigh muscle. . . . Example 12 teaches the finding of hepatitis B [virus] surface antigen in the blood [of] mice injected i.v. with a multifunctional molecular complex comprising a plasmid containing a hepatitis B virus surface antigen gene complexed to a transfer moiety of the invention.

Id., pages 6-7. The examiner found that these examples did not provide sufficient guidance, however, because "in neither case does the expression of the delivered gene result in an alleviation of a symptom of any disease." Id., page 7.

Appellant argues that "[s]ince the claims do not require a therapeutic effect, Applicant need not demonstrate such an effect in order to enable the claimed subject matter." Appeal Brief, page 4. Appellant argues that he "need [] only establish that the application enable[s] one of ordinary skill in the art to make and use a method for transfer[ring] nucleic acid compositions to cells . . . without undue experimentation." Id. Appellant argues that the references cited by the examiner are not applicable because they describe different methods of delivering nucleic acids to cells. Id., page 5. Finally, Appellant relies on a declaration submitted under 37 CFR § 1.132, which is said to provide additional examples of in vivo transfer of nucleic acids using the claimed method. See id., pages 7-9

The examiner bears the initial burden of showing that a claimed method is not enabled. See In re Wright, 999 F.2d 1557, 1561-62, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993) ("[T]he PTO bears an initial burden of setting forth a reasonable explanation as to why it believes that the scope of protection provided by that claim is not adequately enabled by the description of the invention provided in the specification of the application.").

The invention that must be enabled to satisfy § 112 is the invention defined by the claims. See CFMT, Inc. v. Yieldup Int'l Corp., 349 F.3d 1333, 1338, 68 USPQ2d 1940, 1944 (Fed. Cir. 2003) ("Title 35 does not require that a patent disclosure enable one of ordinary skill in the art to make and use a perfected, commercially viable embodiment absent a claim limitation to that effect."). Thus, when the claims are not directed to a method that achieves a therapeutically useful result, achieving such a result is not required for the claims to be enabled.

Here, the claims, as restricted, are directed to a "method for the transfer of a nucleic acid composition [encoding a therapeutic agent] to cells." Thus, while the claims read on gene therapy methods, they do not require producing a clinically effective therapeutic response. Cf. In re Cortright, 165 F.3d 1353, 49 USPQ2d 1464 (Fed. Cir. 1999) (claims to a method of "treating scalp baldness" could be enabled even if the method did not produce a full head of hair).

The examiner argues, however, that the specification must teach those skilled in the art how to use the claimed method to produce a therapeutically useful result because

the only use disclosed for in vivo delivery is [] for therapeutic purposes. . . . Thus, while the specification enables delivery and expression in cells in culture or cells in vitro, the method of delivering has no enabled use for delivery to cells in an animal, patient or subject[;] that is[,] in vivo. There is no evidence that the method results in sufficient delivery of a nucleic acid in vivo to offer a therapeutic effect. The specification offers no use for mere delivery of a therapeutic agent in vivo absent a therapeutic effect.

Examiner's Answer, page 8. As we understand it, the examiner does not dispute that the specification enables those skilled in the art to transfer nucleic acids into cells in

vivo, but she argues that transferring a nucleic acid encoding a therapeutic protein does not produce a useful result unless it confers a therapeutic benefit.

The examiner's reasoning highlights the incorporation into § 112 of the utility requirement of 35 U.S.C. § 101: to be enabled, a claimed method must be disclosed sufficiently to allow those skilled in the art to carry out the recited steps and, in addition, the result of the claimed method must have a specific and substantial utility. See In re Fisher, 421 F.3d 1365, 1378, 76 USPQ2d 1225, 1235 (Fed. Cir. 2005) ("It is well established that the enablement requirement of § 112 incorporates the utility requirement of § 101."); In re Kirk, 376 F.2d 936, 942, 153 USPQ 48, 53 (CCPA 1967) ("[S]urely Congress intended § 112 to pre-suppose full satisfaction of the requirements of § 101. Necessarily, compliance with § 112 requires a description of how to use presently useful inventions, otherwise an applicant would anomalously be required to teach how to use a useless invention.").

The examiner's reasoning is logical but we do not agree that it applies to the instant claims. The specification describes experiments in which exogenous DNA was transferred, using the claimed method, to muscle cells and liver cells in vivo. See pages 77-78. The examiner has not disputed the accuracy of these working examples, but points out that the transferred DNAs did not encode therapeutic proteins and the specification does not describe therapeutically effective gene therapy.

The examiner has cited several references to show that clinical application of gene therapy faced many hurdles in 1994. The examiner has characterized the references as showing that delivering therapeutic genes to cells in vivo and ensuring

adequate expression of the gene products were major areas of unpredictability at the time of filing. See the Examiner's Answer, pages 4-6.

We can accept, for discussion purposes, (1) that the references show that using gene therapy to produce a therapeutically effective result would have required undue experimentation in 1994, and (2) that gene therapy is the only in vivo use disclosed in the specification for the claimed method. Even given those two premises, however, we do not agree that the evidence shows that the claimed method was not enabled as of its effective filing date.

As discussed above, the claims are not directed to a method of carrying out gene therapy, but to a method of transferring nucleic acids into cells. That is, the claimed method is directed to one step in, for example, a gene therapy method. The claimed method is disclosed to overcome some of the problems discussed in the references cited by the examiner. See the specification, pages 2 and 16:

The problems faced by [nonviral vectors or carriers] include . . . means for mediating transport of the genetic material through the cell membrane and into the cytoplasm of the cell; avoidance of degradation within the cell by the reticuloendothelial system; and transport to and through the nuclear membrane into the nucleus of the cell where transcription of the genetic material can take place.

. . .

This multifunctional molecular complex comprises essentially the combination of two key elements, (I) the nucleic acid composition which it is desired to transfer to the target cell, and (II) the transfer moiety, which . . . comprises several components whose function is . . . ii) to overcome the incompatibility arising from the hydrophilic nature of the nucleic acid molecule and the lipophilic nature of the cell membrane so that the former can pass through the latter; and iii) to prevent degradation of the nucleic acid molecule in a lysosome of said target cell, by disrupting the pre-lysosome, endosome formation stage.

The examiner has stated that the in vitro embodiments encompassed by the claims are enabled, and has not disputed the accuracy of the specification's in vivo working examples. There seems to be no dispute, therefore, that the claimed method results in the transfer and expression of nucleic acids in targeted cells. We cannot agree that such a result must provide a therapeutic effect in order to be useful.

A method that overcomes some of the problems plaguing the field of gene therapy would seem to be a useful advance, even if the advance is incremental and does not resolve all of the problems facing the field. Such a method is useful to those skilled in the art even if it is not sufficient, by itself, to allow immediate practice of gene therapy. A method that enhances the efficiency of transfer of nucleic acids to cells in vivo, as the present method is said to do, provides a valid research tool that those skilled in the art could use in carrying out experiments involving transferring nucleic acids to cells in vivo.

The present claims are different from, for example, the invention at issue in In re Fisher, 421 F.3d 1365, 76 USPQ2d 1225 (Fed. Cir. 2005). The applicant in that case claimed expressed sequence tags (ESTs) from genes of unknown function. See id. at 1370, 76 USPQ2d 1231. The court concluded that "the claimed ESTs act as no more than research intermediates that may help scientists to isolate the particular underlying protein-encoding genes and conduct further experimentation on those genes. . . . Accordingly, the claimed ESTs are . . . mere 'object[s] of use-testing,' to wit, objects upon which scientific research could be performed with no assurance that anything useful will be discovered in the end." Id. at 1373, 76 USPQ2d 1231.

The Fisher court considered the applicant's argument that an EST is a research tool, like a microscope, but found the analogy inapt: "[A] microscope has the specific benefit of optically magnifying an object to immediately reveal its structure. One of the claimed ESTs, by contrast, can only be used to detect the presence of genetic material having the same structure as the EST itself. It is unable to provide any information about the overall structure let alone the function of the underlying gene." Id. at 1373, 76 USPQ2d 1231. The court concluded that "Fisher's asserted uses are insufficient to meet the standard for a 'substantial' utility under § 101." Id. at 1373, 76 USPQ2d 1231.

The ESTs at issue in Fisher lacked substantial utility because they were useful only for conducting experiments on the genes of which the ESTs were part; they were not useful for conducting research generally but only for conducting research to learn more about the ESTs themselves and the genes from which they were derived. Here, by contrast, the claimed method is broadly useful for transferring nucleic acids into cells. The instant claims are directed to a completed invention, not a "research intermediate" as in Fisher, that can be used to carry out research using a variety of nucleic acids, cells, and subjects. Thus, the instantly claimed method is a valid research tool that can be used to carry out research in general rather than research limited to discovering information about the claimed invention itself.

Summary

We do not agree with the examiner that enabling the instant claims requires enabling therapeutically effective gene therapy. The specification provides adequate guidance to enable those skilled in the art to use the claimed method to transfer nucleic

acids to cells, and that is all that the claims require. The rejection for lack of enablement is reversed.

REVERSED

Toni R. Scheiner)	
Administrative Patent Judge)	
)	
)	
)	BOARD OF PATENT
Eric Grimes)	
Administrative Patent Judge)	APPEALS AND
)	
)	INTERFERENCES
)	
Richard M. Lebovitz)	
Administrative Patent Judge)	

EG/jlb

Howson and Howson
Cathy A. Kodroff
Suite 210
501 Office Center Drive
Ft Washington, PA 19034

The opinion in support of the decision being entered today was not written for publication and is not binding precedent of the Board.

UNITED STATES PATENT AND TRADEMARK OFFICE

**BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES**

Ex parte NORIMITSU SAITO and MING ZHAO

Appeal No. 2005-1442
Application No. 09/734,786

ON BRIEF

Before ELLIS, SCHEINER, and GRIMES, Administrative Patent Judges.

GRIMES, Administrative Patent Judge.

DECISION ON APPEAL

This appeal involves claims to a method of introducing a nucleic acid into a subject by modifying and transplanting hair follicles. The examiner has rejected the claims as nonenabled. We have jurisdiction under 35 U.S.C. § 134. Because the examiner has not shown that undue experimentation would have been required to practice the claimed method, we reverse.

Background

The specification discloses that "histocultured tissues, including tissues containing hair follicles, can be successfully modified genetically ex vivo and then transplanted successfully into an intact mammalian subject. The success of the

modification is enhanced by treating the histocultured tissues with collagenase prior to genetic modification." Pages 2-3.

The specification states that

[a]lthough it is advantageous to treat the cultured tissue with collagenase in order to enhance the ability of the tissue to accept heterologous nucleic acids, the treatment is not so severe as to destroy completely the integrity of the three-dimensional array.

The three-dimensional histoculture can be assembled from any tissue, including skin, especially skin containing hair follicles, lymphoid tissue, or tumor tissue. The choice of tissue will depend on the nature of the treatment contemplated. . . .

For example, hair follicles are useful recipients of genes intended to affect the growth or quality of hair, but also are able to produce immunogens and other products that may be useful to the organism taken as a whole.

Page 4.

The specification provides a working example in which DNA encoding green fluorescent protein (GFP) was introduced into hair follicles of histocultured mouse skin; the percentage of GFP-expressing hair follicles ranged from 22% to 67%. See pages 11-12. In a second working example, hair follicles in skin samples were transfected with GFP-encoding DNA and grafted onto recipient mice. The results showed that "the percentage of hair follicles with GFP fluorescence in collagenase-treated skin was 5.7 times greater than in hair follicles of untreated skin." Pages 14-15. Fluorescence was detected for at least 10 days after grafting. Figure 3B.

Discussion

1. Claim construction

Claims 1 and 11 are representative of the claims on appeal and read as follows:

1. A method to introduce a nucleic acid molecule into a mammalian subject which method comprises

transplanting into the dermis of said subject at least one hair follicle that has been modified ex vivo to contain said nucleic acid molecule.

11. A method to introduce a nucleic acid molecule into a mammalian subject which method comprises transplanting into the corresponding tissue of said mammal a histocultured intact tissue that has been modified ex vivo to contain said nucleic acid molecule;

wherein said histoculture has been treated with collagenase prior to modifying said tissue with the nucleic acid.

Thus, claim 1 is directed to a method of introducing a nucleic acid into a mammal by modifying a hair follicle ex vivo to contain the nucleic acid and transplanting the hair follicle to the mammal. Claim 1 does not explicitly require that the nucleic acid be expressed or provide any particular benefit to the mammal.

Claim 11 is similar to claim 1 but encompasses treating tissues other than hair follicles; in addition, claim 11 requires that the tissue be treated with collagenase before being modified with the nucleic acid.

2. Enablement

The examiner rejected claims 1-8, 11, 13-15, 17, and 19, all of the claims remaining, under 35 U.S.C. § 112, first paragraph, on the basis that the specification does not enable those skilled in the art to practice the claimed method without undue experimentation. The examiner considered the factors set out in In re Wands, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988), and concluded that

[d]ue to the art recognized unpredictability of achieving therapeutic levels of gene expression following direct or indirect administration of nucleic acids and the lack of guidance provided by the specification for the parameters affecting delivery and expression of therapeutic amounts of DNA into the cells using ex vivo gene transfer into histocultured organs or tissues, it would require undue experimentation to practice the instant invention.

Examiner's Answer, page 10

Appellants argue that the claims are directed to a method of genetically modifying tissues ex vivo and transplanting the modified tissue into a subject, and therefore do not require achieving therapeutic levels of gene expression. Appeal Brief, page 5. Appellants point to the specification's discussion of prior art techniques and working examples as guidance to those skilled in the art. Appellants assert that "[t]he pending claims are fully supported by the ample amount of knowledge available in the relevant art when the present application was filed and the guidance provided in the specification." Id., page 7.

We agree with Appellants that the examiner has not adequately shown that undue experimentation would have been required to practice the claimed method. The examiner bears the initial burden of showing that a claimed invention is nonenabled. See In re Wright, 999 F.2d 1557, 1561-62, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993) ("[T]he PTO bears an initial burden of setting forth a reasonable explanation as to why it believes that the scope of protection provided by that claim is not adequately enabled by the description of the invention provided in the specification of the application.").

"[T]o be enabling, the specification of a patent must teach those skilled in the art how to make and use the full scope of the claimed invention without 'undue experimentation.'" In re Wright, 999 F.2d 1557, 1561, 27 USPQ2d 1510, 1513 (Fed.

Cir. 1993). “That some experimentation may be required is not fatal; the issue is whether the amount of experimentation required is ‘undue.’” In re Vaeck, 947 F.2d 488, 495, 20 USPQ2d 1438, 1444 (Fed. Cir. 1991).

The enablement analysis must be focused on the product or method defined by the claims. “Title 35 does not require that a patent disclosure enable one of ordinary skill in the art to make and use a perfected, commercially viable embodiment absent a claim limitation to that effect.” CFMT, Inc. v. Yieldup Int’l Corp., 349 F.3d 1333, 1338, 68 USPQ2d 1940, 1944 (Fed. Cir. 2003).

Here, the examiner has acknowledged that the claims are not limited to therapeutic methods, but argues that because therapeutic methods are encompassed by the claims, such methods must be enabled in order for the full scope of the claims to be enabled. See the Examiner’s Answer, page 12.

The examiner’s reasoning is logical but not entirely consistent with the case law: enabling the “full scope” of a claim does not necessarily require enabling every embodiment within the claim. See, e.g., Atlas Powder Co. v. E.I. Du Pont De Nemours & Co., 750 F.2d 1569, 1576, 224 USPQ 409, 414 (Fed. Cir. 1984): “Even if some of the claimed combinations were inoperative, the claims are not necessarily invalid. . . . Of course, if the number of inoperative combinations becomes significant, and in effect forces one of ordinary skill in the art to experiment unduly in order to practice the claimed invention, the claims might indeed be invalid.” Atlas Powder concerned claims to a product, not a method as here, but the same principle applies – a claimed method does not lack enablement merely because it cannot be practiced under some circumstances or to achieve some particular result.

In re Cortright, 165 F.3d 1353, 49 USPQ2d 1464 (Fed. Cir. 1999), is instructive. In Cortright, the applicant claimed a method of “treating scalp baldness with an antimicrobial to restore hair growth.” Id. at 1355, 49 USPQ2d at 1465. The Board reversed a rejection for lack of utility, but entered a new rejection for lack of enablement, on the basis that “restor[ing] hair growth” required returning the user’s hair to its original state (a full head of hair). See id. “Because Cortright’s written description discloses results of only ‘three times as much hair growth as two months earlier,’ ‘filling-in some,’ and ‘fuzz,’ the board reasoned, it does not support the breadth of the claims.” Id. at 1358, 49 USPQ2d at 1467.

The court disagreed with the Board’s claim interpretation, holding that “one of ordinary skill would construe this phrase [restoring hair growth] as meaning that the claimed method increases the amount of hair grown on the scalp but does not necessarily produce a full head of hair.” Id. at 1359, 49 USPQ2d at 1468. The court concluded that the claims, so construed, were enabled. Id.

As with the present claims, the claims in Cortright encompassed a method of obtaining results that might be difficult to achieve: here, therapeutically effective gene therapy; in Cortright, complete restoration of hair growth. However, as in Cortright, the present claims do not require that particular result: the present claims require only introducing or delivering a nucleic acid; Cortright’s claims required only some restoration of hair growth.

The court in Cortright did not dispute the Board’s conclusion that completely restoring hair growth using Bag Balm® would require undue experimentation. See id. at 1357, 49 USPQ2d at 1467. The court nonetheless concluded that the claimed method

was not nonenabled merely because it encompassed one difficult-to-achieve outcome. The same reasoning applies here: the examiner may be correct that achieving clinically useful gene therapy using the claimed method would require undue experimentation, but the claims are not nonenabled merely for encompassing that difficult-to-achieve outcome.

The claims are directed to methods of introducing a nucleic acid into a mammalian subject or delivering a nucleic acid to a hair follicle or intact tissue. The examiner has not adequately explained why the specification does not enable those skilled in the art to introduce a nucleic acid into a mammalian subject, or deliver a nucleic acid to a hair follicle or intact tissue, without undue experimentation. We therefore reverse the rejection for nonenablement.

REVERSED

Joan Ellis)	
Administrative Patent Judge)	
)	
)	
)	BOARD OF PATENT
Toni R. Scheiner)	
Administrative Patent Judge)	APPEALS AND
)	
)	INTERFERENCES
)	
Eric Grimes)	
Administrative Patent Judge)	

Morrison & Foerster LLP
3811 Valley Centre Drive
Suite 500
San Diego, CA 92130-2332

pound. But that is not possible in advance, especially when the hypothetical process is only a general one. Thus, a conceived method of preparing some undefined DNA does not define it with the precision necessary to render it obvious over the protein it encodes. We did not state otherwise in *Amgen*. See *Amgen*, 927 F.2d at 1206-09, 18 USPQ2d at 1021-23 (isolated/purified human gene held nonobvious; no conception of gene without envisioning its precise identity despite conception of general process of preparation).

We conclude that, because the applied references do not teach or suggest the claimed cDNA molecules, the final rejection of claims 5 and 7 must be reversed. See also *Bell*, 991 F.2d at 784-85, 26 USPQ2d at 1531-32 (human DNA sequences encoding IGF proteins nonobvious over asserted combination of references showing gene cloning method and complete amino acid sequences of IGFs).

[11] Claims 4 and 6 are of a different scope than claims 5 and 7. As is conceded by Deuel, they generically encompass all DNA sequences encoding human and bovine HBGFs. Written in such a result-oriented form, claims 4 and 6 are thus tantamount to the general idea of all genes encoding the protein, all solutions to the problem. Such an idea might have been obvious from the complete amino acid sequence of the protein, coupled with knowledge of the genetic code, because this information may have enabled a person of ordinary skill in the art to envision the idea of, and, perhaps with the aid of a computer, even identify all members of the claimed genus. The Bohlen reference, however, only discloses a partial amino acid sequence, and thus it appears that, based on the above analysis, the claimed genus would not have been obvious over this prior art disclosure. We will therefore also reverse the final rejection of claims 4 and 6 because neither the Board nor the patent examiner articulated any separate reasons for holding these claims unpatentable apart from the grounds discussed above.

One further matter requires comment. Because Deuel's patent application does not describe how to obtain any DNA except the disclosed cDNA molecules, claims 4 and 6 may be considered to be inadequately sup-

ported by the disclosure of the application. See generally *Amgen Inc. v. Chuqai Pharmaceutical Co.*, 927 F.2d 1200, 1212-14, 18 USPQ2d 1016, 1026-28 (Fed.Cir.) (generic DNA sequence claims held invalid under 35 U.S.C. § 112, first paragraph), cert. denied, 502 U.S. 856, 112 S.Ct. 169, 116 L.Ed.2d 132 (1991); *In re Fisher*, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970) (Section 112 "requires that the scope of the claims must bear a reasonable correlation to the scope of enablement provided by the specification to persons of ordinary skill in the art."). As this issue is not before us, however, we will not address whether claims 4 and 6 satisfy the enablement requirement of § 112, first paragraph, but will leave to the PTO the question whether any further rejection is appropriate.

We have considered the PTO's remaining arguments and find them not persuasive.

CONCLUSION

The Board's decision affirming the final rejection of claims 4-7 is reversed.

REVERSED



In re Miguel F. BRANA, Jose M.C. Berlanga, Marina M. Moset, Erich Schlick and Gerhard Keilhauer.

93-1393.

United States Court of Appeals,
Federal Circuit.

March 30, 1995.

Applicants appealed from decision of the United States Patent and Trademark Office (PTO) Board of Patent Appeals and Interferences, affirming patent examiner's rejections of claims for antitumor compound. The Court of Appeals, Plager, Circuit Judge, held that: (1) claimed specification for antitumor

compound satisfied statutory utility requirement by alleging that compound was more effective in treating lymphocytic leukemia in mice than other known compounds; (2) PTO failed to satisfy its initial burden of challenging presumptively correct assertion of utility; (3) even if one skilled in the art would have reasonably questioned asserted utility of claimed antitumor compound, applicants provided sufficient evidence to convince one of skill in the art of asserted utility; and (4) Food and Drug Administration (FDA) approval is not prerequisite for finding compound useful within meaning of patent laws.

Reversed.

1. Patents \Rightarrow 101(5)

Claim specifications for antitumor compound satisfied statutory utility requirement by alleging that compound was more effective in treating lymphocytic leukemia in mice than other known compounds. 35 U.S.C.A. § 101.

2. Patents \Rightarrow 48

Lymphocytic leukemia tumor models used to study cancer in mice represented specific diseases against which claimed compounds in patent application could be effective, as required to satisfy statutory utility requirement, where cell lines used on models were originally derived from lymphocytic leukemias in mice and would produce that disease once implanted in mice. 35 U.S.C.A. § 101.

3. Patents \Rightarrow 97

Patent and Trademark Office (PTO) has initial burden of challenging presumptively correct assertion of utility in patent disclosure. 35 U.S.C.A. § 101.

4. Patents \Rightarrow 97

Only after Patent and Trademark Office (PTO) provides evidence showing that one of ordinary skill in art would reasonably doubt asserted utility of patented invention does burden shift to applicant to provide rebuttal evidence sufficient to convince such person of invention's asserted utility. 35 U.S.C.A. § 101.

5. Patents \Rightarrow 97

Patent and Trademark Office (PTO) failed to satisfy its initial burden of challenging presumptively correct assertion of utility in application for patent for antitumor compound, where references cited by PTO did not question usefulness of any compound as antitumor agent or provide any other evidence to cause one of skill in the art to question asserted utility of applicants' compounds, but instead discussed therapeutic predictive value of tests used in mice, which were relevant only if applicants were required to prove ultimate value in humans of their asserted utility. 35 U.S.C.A. § 101.

6. Patents \Rightarrow 99

Even if one skilled in the art would have reasonably questioned asserted utility of claimed antitumor compound, applicants provided sufficient evidence to convince one of skill in the art of asserted utility; applicants provided test results showing that several compounds within scope of claims exhibited significant antitumor activity, and prior art disclosed structurally similar compounds which were proven to be effective antitumor agents. 35 U.S.C.A. § 101.

7. Patents \Rightarrow 49

Although minor changes in chemical compounds can radically change their effects on human body, evidence of success in structurally similar compounds is relevant in determining whether one skilled in the art would believe asserted utility.

8. Patents \Rightarrow 46

Food and Drug Administration (FDA) approval is not prerequisite for finding compound useful within meaning of patent laws. Federal Food, Drug, and Cosmetic Act, § 505(i)(1), 21 U.S.C.A. § 355(i)(1); 35 U.S.C.A. §§ 101, 112; 21 C.F.R. §§ 312.21(b), 312.23(a)(5), (a)(8).

9. Patents \Rightarrow 324.5

In reviewing decisions of Patent and Trademark Office (PTO), Court of Appeals traditionally reviews questions of law without deference to views of the agency, and defers to agency with regard to questions of fact unless its findings are clearly erroneous.

10. Patents \S 324.55(1)

When mixed questions of law and fact are before Court of Appeals on appeal from decision of Patent and Trademark Office (PTO), whether Court of Appeals defers, and extent to which it defers to agency's decision, turns on nature of case and nature of judgment. 5 U.S.C.A. \S 706.

Malcolm J. MacDonald, Keil & MacDonald, Washington, DC, argued, for appellant. With him on the brief was Herbert B. Keil. Of counsel was David S. Nagy.

Fred E. McKelvey, Sol., Office of Sol., Arlington, VA, argued, for appellee. With him on the brief were Albin F. Drost, Deputy Sol., Richard E. Schafer, Teddy S. Gron, Joseph G. Piccolo and Richard L. Torczon, Associate Sols.

Before PLAGER, LOURIE, and RADER, Circuit Judges.

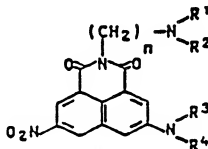
PLAGER, Circuit Judge.

Miguel F. Brana, *et al.* (applicants), appeal the March 19, 1993 decision of the United States Patent and Trademark Office (PTO) Board of Patent Appeals and Interferences (Board), in Appeal No. 92-1196. The Board affirmed the examiner's rejection of claims 10-13 of patent application Serial No. 533,944 under 35 U.S.C. \S 112 ¶ 1 (1988).¹ The examiner's rejection, upon which the Board relied in rendering its decision, was based specifically on a challenge to the utility of the claimed compounds and the amount of experimentation necessary to use the compounds. We conclude the Board erred, and reverse.

I. BACKGROUND

On June 30, 1988, applicants filed patent application Serial No. 213,690 (the '690 application)² directed to 5-nitrobenzo[de]isoquinoline-1,3-dione compounds, for use as anti-

tumor substances, having the following formula:



where n is 1 or 2, R^1 and R^2 are identical or different and are each hydrogen, C_1 - C_6 -alkyl, C_1 - C_6 -hydroxyalkyl, pyrrolidinyl, morpholino, piperidinyl or piperacetyl, and R^3 and R^4 are identical or different and are each hydrogen, C_1 - C_6 -alkyl, C_1 - C_6 -acyl, C_2 - C_7 -alkoxycarbonyl, ureyl, aminocarbonyl or C_2 - C_7 -alkylaminocarbonyl. These claimed compounds differ from several prior art benzo[de]isoquinoline-1,3-dione compounds due to the presence of a nitro group (O_2N) at the 5-position and an amino or other amino group (NR^3R^4) at the 8-position of the isoquinoline ring.

The specification states that these non-symmetrical substitutions at the 5- and 8-positions produce compounds with "a better action and a better action spectrum as antitumor substances" than known benzo[de]isoquinolines, namely those in K.D. Paull *et al.*, *Computer Assisted Structure-Activity Correlations*, Drug Research, 34(II), 1243-46 (1984) (Paull). Paull describes a computer-assisted evaluation of benzo[de]isoquinoline-1,3-diones and related compounds which have been screened for antitumor activity by testing their efficacy *in vivo*³ against two specific implanted murine (i.e., utilizing mice as test subjects) lymphocytic leukemias, P388 and L1210.⁴ These two *in vivo* tests are

Medical Dictionary 798 (25th ed. 1990). *In vitro* means "[i]n an artificial environment, referring to a process or reaction occurring therein, as in a test tube or culture media." *Id.*

1. Unless otherwise noted, all United States Code citations are to the 1988 edition.

2. This is a divisional of patent application Serial No. 110,871 filed October 21, 1987.

3. *In vivo* means "[i]n the living body, referring to a process occurring therein." Steadman's

4. The analysis in Paull consisted of grouping the previously-tested compounds into groups based on common structural features and cross-refer-

widely used by the National Cancer Institute (NCI) to measure the antitumor properties of a compound. Paull noted that one compound in particular, benzo[de]isoquinoline-1,3(2H)dione,5-amino-2(2-dimethyl-aminoethyl [sic]) (hereinafter "NSC 308847"), was found to show excellent activity against these two specific tumor models. Based on their analysis, compound NSC 308847 was selected for further studies by NCI. In addition to comparing the effectiveness of the claimed compounds with structurally similar compounds in Paull, applicants' patent specification illustrates the cytotoxicity of the claimed compounds against human tumor cells, *in vitro*,⁵ and concludes that these tests "had a good action."⁶

The examiner initially rejected applicants' claims in the '690 application as obvious under 35 U.S.C. § 103 in light of U.S. Patent No. 4,614,820, issued to and referred to hereafter as Zee-Cheng et al. Zee-Cheng et al. discloses a benzo[de]isoquinoline compound for use as an antitumor agent with symmetrical substitutions on the 5-position and 8-position of the quinoline ring; in both positions the substitution was either an amino or nitro group.⁷ Although not identical to the applicants' claimed compounds, the examiner noted the similar substitution pattern (i.e., at the same positions on the isoquinoline ring) and concluded that a mixed substitution of the invention therefore would have been obvious in view of Zee-Cheng et al.

In a response dated July 14, 1989, the applicants rebutted the § 103 rejection. Applicants asserted that their mixed disubstituted compounds had unexpectedly better antitumor properties than the symmetrically substituted compounds in Zee-Cheng et al.

encing the various groups, in light of the success rates of the group as a whole, to determine specific compounds that may be effective in treating tumors.

5. See *supra* note 3.

6. The specification does not state the specific type of human tumor cells used in this test.

7. The chemical compound in Zee-Cheng et al. is labeled a 3,6-disubstituted-1,8-naphthalimide and uses different numbering for the positions on the isoquinoline ring. The structure of this compound, however, is identical to that claimed by

In support of this assertion applicants attached the declaration of Dr. Gerhard Keilhauer. In his declaration Dr. Keilhauer reported that his tests indicated that applicants' claimed compounds were far more effective as antitumor agents than the compounds disclosed in Zee-Cheng et al. when tested, *in vitro*, against two specific types of human tumor cells, HEP and HCT-29.⁸ Applicants further noted that, although the differences between the compounds in Zee-Cheng et al. and applicants' claimed compounds were slight, there was no suggestion in the art that these improved results (over Zee-Cheng et al.) would have been expected. Although the applicants overcame the § 103 rejection, the examiner nevertheless issued a final rejection, on different grounds, on September 5, 1989.

On June 4, 1990, applicants filed a continuation application, Serial No. 533,944 (the '944 application), from the above-mentioned '690 application. Claims 10-13, the only claims remaining in the continuation application, were rejected in a final office action dated May 1, 1991. Applicants appealed the examiner's final rejection to the Board.

In his answer to the applicants' appeal brief, the examiner stated that the final rejection was based on 35 U.S.C. § 112 ¶ 1.⁹ The examiner first noted that the specification failed to describe any specific disease against which the claimed compounds were active. Furthermore, the examiner concluded that the prior art tests performed in Paull and the tests disclosed in the specification were not sufficient to establish a reasonable expectation that the claimed compounds had

the applicants except for symmetrical substitutions at the 5-position and the 8-position of the isoquinoline ring. Zee-Cheng et al. teaches identical substitutions of amino or nitro groups while applicants claim a nitro group substitution at the 5-position and an amino group substitution at the 8-position.

8. HEP cells are derived from laryngeal cancer and HCT-29 cells from colon cancer.

9. The examiner's answer noted that the final rejection also could have been made under 35 U.S.C. § 101 for failure to disclose a practical utility.

a practical utility (i.e. antitumor activity in humans).¹⁰

In a decision dated March 19, 1993, the Board affirmed the examiner's final rejection. The three-page opinion, which lacked any additional analysis, relied entirely on the examiner's reasoning. Although noting that it also would have been proper for the examiner to reject the claims under 35 U.S.C. § 101, the Board affirmed solely on the basis of the Examiner's § 112 ¶ 1 rejection. This appeal followed.

II. DISCUSSION

At issue in this case is an important question of the legal constraints on patent office examination practice and policy. The question is, with regard to pharmaceutical inventions, what must the applicant prove regarding the practical utility or usefulness of the invention for which patent protection is sought. This is not a new issue; it is one which we would have thought had been settled by case law years ago.¹¹ We note the Commissioner has recently addressed this question in his Examiner Guidelines for Biotech Applications, *see* 60 Fed.Reg. 97 (1995); 49 Pat.Trademark & Copyright J. (BNA) No. 1210, at 234 (Jan. 5, 1995).

The requirement that an invention have utility is found in 35 U.S.C. § 101: "Whoever invents . . . any new and useful . . . composition of matter . . . may obtain a patent therefor . . ." (emphasis added). It is also implicit in § 112 ¶ 1, which reads:

10. The examiner subsequently filed two supplemental answers in response to arguments raised by the applicants in supplemental reply briefs.

11. *See, e.g., Cross v. Iizuka*, 753 F.2d 1040, 224 USPQ 739 (Fed.Cir.1985); *In re Langer*, 503 F.2d 1380, 183 USPQ 288 (CCPA 1974); *In re Krimmel*, 292 F.2d 948, 130 USPQ 215 (CCPA 1961); *In re Bergel*, 292 F.2d 958, 130 USPQ 205 (CCPA 1961).

12. This court's predecessor has determined that absence of utility can be the basis of a rejection under both 35 U.S.C. § 101 and § 112 ¶ 1. *In re Jolles*, 628 F.2d 1322, 1326 n. 11, 206 USPQ 885, 889 n. 11 (CCPA 1980); *In re Fouche*, 439 F.2d 1237, 1243, 169 USPQ 429, 434 (CCPA 1971) ("[I]f such compositions are in fact useless, appellant's specification cannot have taught how to use them."). Since the Board affirmed the ex-

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Obviously, if a claimed invention does not have utility, the specification cannot enable one to use it.

As noted, although the examiner and the Board both mentioned § 101, and the rejection appears to be based on the issue of whether the compounds had a practical utility, a § 101 issue, the rejection according to the Board stands on the requirements of § 112 ¶ 1. It is to that provision that we address ourselves.¹² The Board gives two reasons for the rejection;¹³ we will consider these in turn.

1.

[1] The first basis for the Board's decision was that the applicants' specification failed to disclose a specific disease against which the claimed compounds are useful, and therefore, absent undue experimentation, one of ordinary skill in the art was precluded from using the invention. *See Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed.Cir.1986), *cert. denied*, 480 U.S. 947, 107 S.Ct. 1606, 94 L.Ed.2d 792 (1987). In support, the Commissioner argues that the disclosed uses in

aminer's rejection based solely on § 112 ¶ 1, however, our review is limited only to whether the application complies with § 112 ¶ 1.

13. The Board's decision did not expressly make any independent factual determinations or legal conclusions. Rather, the Board stated that it "agree[d] with the examiner's well reasoned, well stated and fully supported by citation of relevant precedent position in every particular, and any further comment which we might add would be redundant." *Ex parte Brana et al.*, No. 92-1196 (Bd.Pat.App. & Int. March 19, 1993) at 2-3. Therefore, reference in this opinion to Board findings are actually arguments made by the examiner which have been expressly adopted by the Board.

the '944 application, namely the "treatment of diseases" and "antitumor substances," are similar to the nebulous disclosure found insufficient in *In re Kirk*, 376 F.2d 936, 153 USPQ 48 (CCPA 1967). This argument is not without merit.

In *Kirk* applicants claimed a new class of steroid compounds. One of the alleged utilities disclosed in the specification was that these compounds possessed "high biological activity." *Id.* at 938, 153 USPQ at 50. The specification, however, failed to disclose which biological properties made the compounds useful. Moreover, the court found that known specific uses of similar compounds did not cure this defect since there was no disclosure in the specification that the properties of the claimed compounds were the same as those of the known similar compounds. *Id.* at 942, 153 USPQ at 53. Furthermore, it was not alleged that one of skill in the art would have known of any specific uses, and therefore, the court concluded this alleged use was too obscure to enable one of skill in the art to use the claimed invention. See also *Kawai v. Metlesies*, 480 F.2d 880, 178 USPQ 158 (CCPA 1973).

Kirk would potentially be dispositive of this case were the above-mentioned language the only assertion of utility found in the '944 application. Applicants' specification, however, also states that the claimed compounds have "a better action and a better action spectrum as antitumor substances" than known compounds, specifically those analyzed in Paull. As previously noted, see *supra* note 4, Paull grouped various benzo(de)isoquinoline-1,3-diones, which had previously been tested *in vivo* for antitumor activity against two lymphocytic leukemia tumor models (P388 and L1210), into various structural classifications and analyzed the test results of the groups (i.e. what percent of the compounds in the particular group showed success against the tumor models). Since one of the tested compounds, NSC 308847, was found to be highly effective against these two lymphocytic leukemia tumor models,¹⁴ applicants' favorable compari-

son implicitly asserts that their claimed compounds are highly effective (i.e. useful) against lymphocytic leukemia. An alleged use against this particular type of cancer is much more specific than the vaguely intimated uses rejected by the courts in *Kirk* and *Kawai*. See, e.g., *Cross v. Iizuka*, 753 F.2d at 1048, 224 USPQ at 745 (finding the disclosed practical utility for the claimed compounds—the inhibition of thromboxane synthetase in human or bovine platelet microsomes—sufficiently specific to satisfy the threshold requirement in *Kirk* and *Kawai*.)

[2] The Commissioner contends, however, that P388 and L1210 are not diseases since the only way an animal can get sick from P388 is by a direct injection of the cell line. The Commissioner therefore concludes that applicants' reference to Paull in their specification does not provide a specific disease against which the claimed compounds can be used. We disagree.

As applicants point out, the P388 and L1210 cell lines, though technically labeled tumor models, were originally derived from lymphocytic leukemias in mice. Therefore, the P388 and L1210 cell lines do represent actual specific lymphocytic tumors; these models will produce this particular disease once implanted in mice. If applicants were required to wait until an animal naturally developed this specific tumor before testing the effectiveness of a compound against the tumor *in vivo*, as would be implied from the Commissioner's argument, there would be no effective way to test compounds *in vivo* on a large scale.

We conclude that these tumor models represent a specific disease against which the claimed compounds are alleged to be effective. Accordingly, in light of the explicit reference to Paull, applicants' specification alleges a sufficiently specific use.

2.

[3,4] The second basis for the Board's rejection was that, even if the specification did allege a specific use, applicants failed to

and Colon C872.

14. Paull also found NSC 308847 to be effective against two other test models, B16 melanoma

prove that the claimed compounds are useful. Citing various references,¹⁵ the Board found, and the Commissioner now argues, that the tests offered by the applicants to prove utility were inadequate to convince one of ordinary skill in the art that the claimed compounds are useful as antitumor agents.¹⁶

This court's predecessor has stated:

[A] specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented *must* be taken as in compliance with the enabling requirement of the first paragraph of § 112 *unless* there is reason to doubt the objective truth of the statements contained therein which *must* be relied on for enabling support.

In re Marzocchi, 439 F.2d 220, 223, 169 USPQ 367, 369 (CCPA 1971). From this it follows that the PTO has the initial burden of challenging a presumptively correct assertion of utility in the disclosure. *Id.* at 224, 169 USPQ at 370. Only after the PTO provides evidence showing that one of ordinary skill in the art would reasonably doubt the asserted utility does the burden shift to the applicant to provide rebuttal evidence sufficient to convince such a person of the invention's asserted utility. See *In re Bundy*, 642 F.2d 430, 433, 209 USPQ 48, 51 (CCPA 1981).¹⁷

[5] The PTO has not met this initial burden. The references cited by the Board, Pazdur and Martin,¹⁸ do not question the usefulness of any compound as an antitumor agent or provide any other evidence to cause one of skill in the art to question the asserted

utility of applicants' compounds. Rather, these references merely discuss the therapeutic predictive value of *in vivo* murine tests—relevant only if applicants must prove the ultimate value in humans of their asserted utility. Likewise, we do not find that the nature of applicants' invention alone would cause one of skill in the art to reasonably doubt the asserted usefulness.

The purpose of treating cancer with chemical compounds does not suggest an inherently unbelievable undertaking or involve implausible scientific principles. *In re Jolles*, 628 F.2d at 1327, 206 USPQ at 890. Modern science has previously identified numerous successful chemotherapeutic agents. In addition, the prior art, specifically Zee Cheng et al., discloses structurally similar compounds to those claimed by the applicants which have been proven *in vivo* to be effective as chemotherapeutic agents against various tumor models.

Taking these facts—the nature of the invention and the PTO's proffered evidence—into consideration we conclude that one skilled in the art would be without basis to reasonably doubt applicants' asserted utility on its face. The PTO thus has not satisfied its initial burden. Accordingly, applicants should not have been required to substantiate their presumptively correct disclosure to avoid a rejection under the first paragraph of § 112. See *In re Marzocchi*, 439 F.2d at 224, 169 USPQ at 370.

[6] We do not rest our decision there, however. Even if one skilled in the art

15. See Pazdur et al., *Correlation of Murine Antitumor Models in Predicting Clinical Drug Activity in Non-Small Cell Lung Cancer: A Six Year Experience*, 3 Proceedings Am.Soc.Clin.Oncology 219 (1984); Martin et al., *Role of Murine Tumor Models in Cancer Research*, 46 Cancer Research 2189 (April 1986).

16. As noted, this would appear to be a § 101 issue, rather than § 112.

17. See also *In re Novak*, 306 F.2d 924, 928, 134 USPQ 335, 337 (CCPA 1962) (stating that it is proper for the examiner to request evidence to substantiate an asserted utility unless one with ordinary skill in the art would accept the allegations as obviously valid and correct); *In re Chi-*

lowsky, 229 F.2d 457, 462, 108 USPQ 321, 325 (CCPA 1956) ("[W]here the mode of operation alleged can be readily understood and conforms to the known laws of physics and chemistry . . . no further evidence is required."). But see *In re Marzocchi*, 439 F.2d at 223, 169 USPQ at 369-70 ("In the field of chemistry generally there may be times when the well-known unpredictability of chemical reactions will alone be enough to create a reasonable doubt as to the accuracy of a particular broad statement put forward as enabling support for a claim. This will especially be the case where the statement is, on its face, contrary to generally accepted scientific principles.").

18. See *supra* note 15.

would have reasonably questioned the asserted utility, i.e., even if the PTO met its initial burden thereby shifting the burden to the applicants to offer rebuttal evidence, applicants proffered sufficient evidence to convince one of skill in the art of the asserted utility. In particular, applicants provided through Dr. Kluge's declaration¹⁹ test results showing that several compounds within the scope of the claims exhibited significant antitumor activity against the L1210 standard tumor model *in vivo*. Such evidence alone should have been sufficient to satisfy applicants' burden.

[7] The prior art further supports the conclusion that one skilled in the art would be convinced of the applicants' asserted utility. As previously mentioned, prior art—Zee Cheng et al. and Paull—disclosed structurally similar compounds which were proven *in vivo* against various tumor models to be effective as chemotherapeutic agents. Although it is true that minor changes in chemical compounds can radically alter their effects on the human body, *Kawai*, 480 F.2d at 891, 178 USPQ at 167, evidence of success in structurally similar compounds is relevant in determining whether one skilled in the art would believe an asserted utility. See *Rey-Bellet v. Engelhardt*, 493 F.2d 1380, 181 USPQ 453 (CCPA 1974); *Kawai*, 480 F.2d 880, 178 USPQ 158.

The Commissioner counters that such *in vivo* tests in animals are only preclinical tests to determine whether a compound is suitable for processing in the second stage of testing, by which he apparently means *in vivo* testing in humans, and therefore are not reasonably predictive of the success of the claimed compounds for treating cancer in humans.²⁰ The

Commissioner, as did the Board, confuses the requirements under the law for obtaining a patent with the requirements for obtaining government approval to market a particular drug for human consumption. See *Scott v. Finney*, 34 F.3d 1058, 1063, 32 USPQ2d 1115, 1120 (Fed.Cir.1994) ("Testing for the full safety and effectiveness of a prosthetic device is more properly left to the Food and Drug Administration (FDA). Title 35 does not demand that such human testing occur within the confines of Patent and Trademark Office (PTO) proceedings.").

Our court's predecessor has determined that proof of an alleged pharmaceutical property for a compound by statistically significant tests with standard experimental animals is sufficient to establish utility. *In re Krimmel*, 292 F.2d 948, 953, 130 USPQ 215, 219 (CCPA 1961); see also *In re Bergel*, 292 F.2d 958, 130 USPQ 205 (CCPA 1961). In concluding that similar *in vivo* tests were adequate proof of utility the court in *In re Krimmel* stated:

We hold as we do because it is our firm conviction that one who has taught the public that a compound exhibits some desirable pharmaceutical property in a standard experimental animal has made a significant and useful contribution to the art, even though it may eventually appear that the compound is without value in the treatment in humans.

Krimmel, 292 F.2d at 953, 130 USPQ at 219. Moreover, NCI apparently believes these tests are statistically significant because it has explicitly recognized both the P388 and L1210 murine tumor models as standard screening tests for determining whether new

disclosure enabling, but instead goes to prove that the disclosure was in fact enabling when filed (i.e., demonstrated utility).

19. The declaration of Michael Kluge was signed and dated June 19, 1991. This declaration listed test results (i.e. antitumor activity) of the claimed compounds, *in vivo*, against L1210 tumor cells and concluded that these compounds would likely be clinically useful as anti-cancer agents. Enablement, or utility, is determined as of the application filing date. *In re Glass*, 492 F.2d 1228, 1232, 181 USPQ 31, 34 (CCPA 1974). The Kluge declaration, though dated after applicants' filing date, can be used to substantiate any doubts as to the asserted utility since this pertains to the accuracy of a statement already in the specification. *In re Marzocchi*, 439 F.2d at 224 n. 4, 169 USPQ at 370 n. 4. It does not render an insufficient

20. We note that this discussion is relevant to the earlier discussion as well. If we were to conclude that these *in vivo* tests are insufficient to establish usefulness for the claimed compounds, that would bear on the issue of whether one skilled in the art would, in light of the structurally similar compounds in Paull and Zee Cheng et al., have cause to doubt applicants' asserted usefulness for the compounds.

compounds may be useful as antitumor agents.

In the context of this case the Martin and Pazdur references, on which the Commissioner relies, do not convince us otherwise. Pazdur only questions the reliability of the screening tests against lung cancer; it says nothing regarding other types of tumors. Although the Martin reference does note that some laboratory oncologists are skeptical about the predictive value of *in vivo* murine tumor models for human therapy, Martin recognizes that these tumor models continue to contribute to an increasing human cure rate. In fact, the authors conclude that this perception (i.e. lack of predictive reliability) is not tenable in light of present information.

On the basis of animal studies, and controlled testing in a limited number of humans (referred to as Phase I testing), the Food and Drug Administration may authorize Phase II clinical studies. See 21 U.S.C. § 355(f)(1); 21 C.F.R. § 312.23(a)(5), (a)(8) (1994). Authorization for a Phase II study means that the drug may be administered to a larger number of humans, but still under strictly supervised conditions. The purpose of the Phase II study is to determine primarily the safety of the drug when administered to a larger human population, as well as its potential efficacy under different dosage regimens. See 21 C.F.R. § 312.21(b).

[8] FDA approval, however, is not a prerequisite for finding a compound useful within the meaning of the patent laws. *Scott*, 34 F.3d 1068, 1063, 32 USPQ2d 1115, 1120. Usefulness in patent law, and in particular in the context of pharmaceutical inventions, necessarily includes the expectation of further research and development. The stage at which an invention in this field becomes useful is well before it is ready to be administered to humans. Were we to require Phase II testing in order to prove utility, the associated costs would prevent many companies from obtaining patent protection on promising new inventions, thereby eliminating an incentive to pursue, through research and development, potential cures in many crucial areas such as the treatment of cancer.

In view of all the foregoing, we conclude that applicants' disclosure complies with the requirements of 35 U.S.C. § 112 ¶1.

3.

[9] The Commissioner takes this opportunity to raise the question of this court's standard of review when deciding cases on appeal from the PTO. Traditionally we have recited our standard of review to be, with regard to questions of law, that review is without deference to the views of the Agency, *In re Donaldson*, 16 F.3d 1189, 1192, 29 USPQ2d 1845, 1848 (Fed.Cir.1994) (in banc), *In re Caveney*, 761 F.2d 671, 674, 226 USPQ 1, 3 (Fed.Cir.1985), and with regard to questions of fact, we defer to the Agency unless its findings are "clearly erroneous." See, e.g., *In re Baxter Travenol Labs*, 952 F.2d 388, 21 USPQ2d 1281 (Fed.Cir.1991); *In re Woodruff*, 919 F.2d 1575, 16 USPQ2d 1934 (Fed.Cir.1990); *In re De Blauwe*, 736 F.2d 699, 222 USPQ 191 (Fed.Cir.1984).

[10] With regard to judgment calls, those questions that fall "[s]omewhere near the middle of the fact-law spectrum," this court has recognized "the falseness of the fact-law dichotomy, since the determination at issue, involving as it does the application of a general legal standard to particular facts, is probably most realistically described as neither of fact nor law, but mixed." *Campbell v. Merit Systems Protection Board*, 27 F.3d 1560, 1565 (Fed.Cir.1994). When these questions of judgment are before us, whether we defer, and the extent to which we defer, turns on the nature of the case and the nature of the judgment. *Id.* ("Characterization therefore must follow from an *a priori* decision as to whether deferring . . . is sound judicial policy. We would be less than candid to suggest otherwise.")

The Commissioner contends that the appropriate standard of review for this court regarding questions of law, of fact, and mixed questions of law and fact, coming to us from the PTO is found in the Administrative Procedure Act (APA) at 5 U.S.C. § 706. The standard set out there is that "[t]he reviewing court shall . . . hold unlawful and set aside agency action, findings, and conclusions found to be—(A) arbitrary, capricious, an

abuse of discretion, or otherwise not in accordance with law; . . . (E) unsupported by substantial evidence. . . ." The Commissioner is of the view that the stated standard we now use, which is the traditional standard of review for matters coming from a trial court, is not appropriate for decisions coming from an agency with presumed expertise in the subject area, and is not in accord with law.²¹

Applicants argue that by custom and tradition, recognized by the law of this court, the standard of review we have applied, even though inconsistent with the standard set forth in the APA, nevertheless is a permissible standard. In our consideration of this issue, there is a reality check: would it matter to the outcome in a given case which formulation of the standard a court articulates in arriving at its decision? The answer no doubt must be that, even though in some cases it might not matter, in others it would, otherwise the lengthy debates about the meaning of these formulations and the circumstances in which they apply would be unnecessary.

A preliminary question, then, is whether this is one of those cases in which a difference in the standard of review would make a difference in the outcome. The ultimate issue is whether the Board correctly applied the § 112 ¶ 1 enablement mandate and its implicit requirement of practical utility, or perhaps more accurately the underlying requirement of § 101, to the facts of this case. As we have explained, the issue breaks down into two subsidiary issues: (1) whether a person of ordinary skill in the art would conclude that the applicants had sufficiently described particular diseases addressed by the invention, and (2) whether the Patent Act supports a requirement that makes human testing a prerequisite to patentability under the circumstances of this case.

The first subsidiary issue, whether the application adequately described particular diseases, calls for a judgment about what the various representations and discussions contained in the patent application's specification

would say to a person of ordinary skill in the art. We have considered that question carefully, and, for the reasons we explained above in some detail, we conclude that the Board's judgment on this question was erroneous. Our conclusion rests on our understanding of what a person skilled in the art would gather from the various art cited, and from the statements in the application itself. We consider the Board's error to be sufficiently clear that it is reversible whether viewed as clear error or as resulting in an arbitrary and capricious decision.

The second subsidiary issue, whether human testing is a prerequisite to patentability, is a pure question of law: what does the practical utility requirement mean in a case of this kind. Under either our traditional standard or under the APA standard no deference is owed the Agency on a question of law, and none was accorded.

If the question concerning the standard of review, raised by the Commissioner, is to be addressed meaningfully, it must arise in a case in which the decision will turn on that question, and, recognizing this, the parties fully brief the issue. This is not that case. We conclude that it is not necessary to the disposition of this case to address the question raised by the Commissioner; accordingly, we decline the invitation to do so.

III. CONCLUSION

The Board erred in affirming the examiner's rejection under 35 U.S.C. § 112 ¶ 1. The decision is reversed.

REVERSED.



21. Congress enacted the Administrative Procedure Act (APA) on June 11, 1946. See 1 Kenneth Culp Davis, *Administrative Law Treatise*, § 1:7 (2d ed. 1978). The APA sets forth a framework for administrative agency procedure and pro-

vides judicial review for persons adversely affected by final agency actions. Chapter 7, codified at 5 U.S.C. § 701-706, contains the APA judicial review provisions, including the standard of review provision quoted above.

the art along with Martin for almost eight years before the filing date of the present application.

The board's decision is affirmed.

Affirmed.



58 CCPA

**Application of Alfred MARZOCCHI and
Richard C. Horton.**

Patent Appeal No. 8431.

United States Court of Customs
and Patent Appeals.

April 15, 1971.

Appeal from decision of the patent office board of appeals which affirmed final rejection of claims 5, 6, 11 and 12 of application, serial No. 470,618, involving technique for improving adhesion characteristics between glass fibers and vinyl polymer resins. The Court of Customs and Patent Appeals, Baldwin, J., held that claims 5 and 11, teaching use of monomeric vinyl pyrrolidone, were obvious in light of reference teaching use of polymeric vinyl pyrrolidone, but that claims 6 and 12, reciting use of polyethyleneamine, were supported by disclosure which was in compliance with requirements of specification statute despite the breadth of the claim, where record contained insufficient grounds for questioning the accuracy of teaching that any polyethyleneamine would function to accomplish the asserted result.

Affirmed in part and reversed in part.

1. Patents \Rightarrow 18

In connection with patent application involving technique for improving adhesion characteristics between glass fibers and vinyl polymer resins, claims 5 and 11, teaching use of monomeric

vinyl pyrrolidone were obvious in light of reference teaching use of polymeric vinyl pyrrolidone. 35 U.S.C.A. § 103.

2. Patents \Rightarrow 18

Inference of fact that, to one possessing the ordinary level of skill in the art, it would be obvious to try particular composition may at times be enough to justify drawing the ultimate conclusion of law that the claimed subject matter as a whole would have been obvious. 35 U.S.C.A. § 103.

3. Patents \Rightarrow 101(4)

Claims 6 and 12 of patent application relating to technique for improving adhesion characteristics between glass fibers and vinyl polymer resins, which recited use of "polyethyleneamine" were supported by disclosure which was in compliance with specification statute despite contention as to excessive breadth of the disputed term, where record contained insufficient grounds for questioning accuracy of teaching that any polyethyleneamine would function to accomplish the asserted result. 35 U.S.C.A. § 112.

4. Patents \Rightarrow 101(4)

Where generic term is recited in patent application, the only relevant concern of the patent office under specification statute should be the truth of the assertion that any member of the class will accomplish the desired result, not the breadth of the term. 35 U.S.C.A. § 112.

5. Patents \Rightarrow 101(4)

Specification disclosure which contains teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as in compliance with the enabling requirement of the specification statute unless there is reason to doubt the objective truth of statements contained in the specification which must be relied on for enabling support. 35 U.S.C.A. § 112.

6. Patents \Rightarrow 113(7)

Unpredictability of chemical reactions alone may be enough to create

reasonable doubt as to accuracy of particular broad statement put forward as enabling support for claim, especially where the statement is, on its face, contrary to generally accepted scientific principles, but it is incumbent upon the patent office, when rejection is made on this basis, to explain why it doubts the truth or accuracy of the statement. 35 U.S.C.A. § 112.

7. Patents \approx 101(4)

In considering accuracy of specification, pertinent references are not necessarily prior art references. 35 U.S.C.A. § 112.

Herman Hersh, McDougall, Hersh, Scott & Ladd, Chicago, Ill., attorney of record, for appellant. Staelin & Overman, Toledo, Ohio, George A. Degnan, Arlington, Va., of counsel.

S. Wm. Cochran, Washington, D. C., for the Commissioner of Patents. Fred W. Sherling, Washington, D. C., of counsel.

Before RICH, ALMOND, BALDWIN and LANE, Judges, and DURFEE, Judge, United States Court of Claims, sitting by designation.

BALDWIN, Judge.

This is an appeal from the decision of the Patent Office Board of Appeals which affirmed the final rejection of claims 5 and 11 of appellants' application¹ under 35 U.S.C. § 103 as unpatentable in view of Werner² and of claims 6 and 12 under 35 U.S.C. § 112 as being based on an inadequate disclosure. Claims 4 and 10 stand allowed.

THE INVENTION

The subject matter of the claims on appeal involves a technique for improving the adhesion characteristics between glass fibers and vinyl polymer resins.

Claim 5 is representative and reads as follows:

5. In the combination of glass fibers and a vinyl polymer resin composition present as a coating on the glass fiber surfaces, the improvement which comprises mixing the vinyl polymer resin, prior to coating of the glass fibers, with an amine compound in an amount corresponding to 2-10% by weight of the vinyl polymer resin, and in which the amine compound is monomeric vinyl pyrrolidone.

Claim 11 is drawn to the same concept as claim 5, but defines the invention as "a method of producing glass fibers coated with polyvinyl resin strongly bonded to the glass fiber surfaces." Claims 6 and 12 differ from claims 5 and 11 respectively solely in the recitation of "polyethyleneamine" as the critical "amine compound" additive.

THE SECTION 103 REJECTION

Claims 5 and 11 were rejected "as obvious in the sense of 35 USC 103 over Werner." Werner, the sole reference relied upon here, is addressed to the improvement in the bonding relationship between glass and polyvinyl halide resins. The pertinent disclosure is as follows [emphasis added]:

I have found that polyvinyl halide resins may be successfully modified so as to obtain excellent glass adhesion by employing a mixture of a polyvinyl halide and a polymer of N-vinyl pyrrolidone. By employing a mixture containing from 80 to 97% of a polyvinyl halide and from 20 to 3% of a polymer of N-vinyl pyrrolidone, which term includes homopolymers of vinyl pyrrolidone and copolymers with other polymerizable monomers, a composition is obtained having extremely high adhesion to all glass surfaces.

On the basis of this teaching the examiner took the position, accepted by the

1. Serial No. 470,618, filed July 8, 1965, for "Fiber Coatings—Nitrogen Compounds for Improving Adhesion of Vinyl Polymers to Glass" as a continuation-in-part of Serial No. 96,106, filed March 16, 1961.

2. U. S. Patent No. 2,853,465, issued September 23, 1958.

board, that the claimed use of *monomeric* vinyl pyrrolidone rather than Werner's *polymeric* vinyl pyrrolidone would be obvious to one of ordinary skill in the art since Werner's teaching would indicate to "one skilled in the art * * * that it is the vinyl pyrrolidone moiety that is enhancing the adhesion." It was also suggested by the examiner that since the claims recite no temperature conditions for the coating operation and since monomers polymerize when heated, the claims could possibly cover circumstances wherein the monomer is polymerized during application. The board appears to have accepted this suggestion and to have extended it even further. It stated:

All of Werner's examples specify heating at elevated temperatures (110°C.-130°C., 165°C., 325°F., 350°F) with and without elevated pressures. Appellants' specification says nothing about retaining the vinyl pyrrolidone in monomeric form, much less anything about "maximizing adhesion" by preventing polymerization. Indeed, the very designation of the vinyl pyrrolidone as a "monomeric" material introduced into a polymer system for the purpose of altering the properties of such system implies subsequent polymerization of the monomer. Appellants' further argument that the monomer has entirely different capabilities and solubilities than the polymer is also unpersuasive.

Appellants' position on appeal in response to these assertions by the examiner and board is largely to stress again the "marked difference between the properties and characteristics of a polymer as compared to a monomer," and to object to the "purely conjectural" assertion that the monomer polymerizes in the

coating after it is applied. Additionally, appellants make the following contention:

Even if it were assumed that appellants' monomeric vinyl pyrrolidone is polymerized when present in the polyvinyl chloride coating, there is no teaching or suggestion in Werner that the use of monomeric vinyl pyrrolidone has any efficacy whatsoever in compositions of the type disclosed and claimed. The basis suggested by the Patent Office for the rejection is tantamount to the allegation it would be "obvious to try" the monomer. This "test" of obviousness has been frequently repudiated by this court.

[1] The sole issue is, of course, whether the Werner teaching does suggest to a person having ordinary skill in this art that the use of monomeric vinyl pyrrolidone would have the efficacy indicated in the appealed claims. We agree with appellants that whether the monomer polymerizes is irrelevant, at least in this regard. What is relevant, however, and here determinative, is the examiner's assertion that the Werner teaching would suggest that it is the vinyl pyrrolidone moiety alone and not some other characteristic peculiar to a polymer which is efficacious in producing the desired adhesion enhancement.³ In the absence of anything to rebut this assertion, which is reasonable on its face, we are constrained to accept it as fact. The inferences which follow from such fact, *i. e.*, that the monomer would possess this same characteristic and that one of ordinary skill would recognize such fact, are inescapable.

[2] It is acknowledged that the above line of reasoning may be viewed as being tantamount to drawing the inference that, to one possessing the ordinary level of skill in this art, it would be "obvious

3. Indeed, the reasonableness of such an assertion is confirmed by the very disclosure contained in appellants' application which indicates that efficacious adhesion enhancers are those "organic nitrogenous compounds which are characterized both by an organic constitution which is com-

patible with the vinyl polymers and by a polarity expressed in the nitrogen function." As also pointed out by appellants in their brief (about which more will be said later), the nature of the present invention resides in the use of *amine* compounds, broadly, as adhesion enhancers.

to try" the monomer. Nevertheless, such an *inference of fact may*, at times, be enough to justify drawing the ultimate *conclusion of law* that the claimed subject matter as a whole would have been obvious under section 103. We are satisfied that the circumstances of this case justify an initial conclusion of obviousness. Since the record before us contains nothing to rebut that conclusion, the decision with regard to claims 5 and 11 must be affirmed.

THE SECTION 112 REJECTION

Claims 6 and 12, which recite the use of "polyethylenamine" as the adhesion enhancer, were criticized by the examiner as being based on a disclosure which was not enabling under the first paragraph of 35 U.S.C. § 112. The board affirmed his rejection of those claims with the following comment.

The term is obviously generic to a considerable number of compounds varying in the number of ethylene groups, the number of amine groups and the relationship of the polyethylene groups to the amine groups, and accordingly does not provide a reasonable guide for those seeking to improve the adherence of vinyl resins to glass.

[3] We will reverse the board's decision on this rejection since we are unable to find sufficient justification for the holding that appellants' disclosure is not enabling.

[4] Turning specifically to the objections noted by the board as indicated above, it appears that these comments indicate nothing more than a concern over the *breadth* of the disputed term. If we are correct, then the relevance of this concern escapes us. It has never been contended that appellants, when they included the disputed term in their specification, intended only to indicate a single compound. Accepting, therefore, that the term is a generic one, its recita-

tion must be taken as an assertion by appellants that all of the "considerable number of compounds" which are included within the generic term would, as a class, be operative to produce the asserted enhancement of adhesion characteristics. The only relevant concern of the Patent Office under these circumstances should be over the *truth* of any such assertion. The first paragraph of § 112 requires nothing more than objective enablement. How such a teaching is set forth, either by the use of illustrative examples or by broad terminology, is of no importance.

[5] As a matter of Patent Office practice, then, a specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented *must* be taken as in compliance with the enabling requirement of the first paragraph of § 112 *unless* there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support. Assuming that sufficient reason for such doubt does exist, a rejection for failure to teach how to make and/or use will be proper on that basis; such a rejection can be overcome by suitable proofs indicating that the teaching contained in the specification is truly enabling.

[6, 7] In the field of chemistry generally, there may be times when the well-known unpredictability of chemical reactions will alone be enough to create a reasonable doubt as to the accuracy of a particular broad statement put forward as enabling support for a claim. This will especially be the case where the statement is, on its face, contrary to generally accepted scientific principles. Most often, additional factors, such as the teachings in pertinent references,⁴ will be available to substantiate any doubts that the asserted scope of objective enable-

in the specification, not whether that statement had been made before.

4. Not necessarily *prior art* references, it should be noted, since the question would be regarding the *accuracy* of a statement

ment is in fact commensurate with the scope of protection sought and to support any demands based thereon for proof. In any event, it is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain *why* it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement. Otherwise, there would be no need for the applicant to go to the trouble and expense of supporting his presumptively accurate disclosure. Cf. *In re Gazave*, 379 F.2d 973, 54 CCPA 1524 (1967); *In re Chilowsky*, 229 F.2d 457, 43 CCPA 775 (1956).

In the present case, the circumstances we see do not support the reasonableness of any doubts which the Patent Office might have had concerning the adequacy of appellants' specification disclosure to support these claims. In fact, those circumstances tend to strengthen rather than weaken appellants' claim to the breadth of protection they seek. In the first place, it has not been asserted by the Patent Office that the chemical properties of known polyethylenamines vary to such an extent that it would not be expected by one of ordinary skill in this art that any such compound would possess the necessary capability of enhancing adhesion. Additionally, we note that polyethyleneamine is listed in appellants' specification as being only one of a much larger class of amine compounds possessing this necessary characteristic. Finally, we recognize (as did

the examiner) the generic nature of appellants' broader concept, *i. e.*, that the desired property of adhesion enhancement stems largely from the amine moiety. It does appear that variation of certain of the secondary factors mentioned by the examiner, such as molecular weight or proportion of ethylene groups, might influence to some degree or even mask the essential "amine" property of the polyethyleneamine or its obviously equally essential compatibility with vinyl polymers. However, we see no basis to conclude that the ready avoidance of this result would not be within the level of ordinary skill in this art. Compare *In re Skrivan*, 427 F.2d 801, 57 CCPA 1201 (1970).

Taking all these circumstances into consideration, we are constrained to conclude that the record before us contains insufficient grounds for questioning the accuracy of appellants' teaching that *any* polyethyleneamine (obviously excepting those whose essential "amine" characteristics and compatibility with vinyl polymers would be masked by the secondary factors mentioned) will function to accomplish the asserted result. It follows that claims 6 and 12 must be held to be supported by a disclosure which is in compliance with the requirements of the first paragraph of 35 U.S.C. § 112.

SUMMARY

The decision of the board regarding claims 5 and 11 is affirmed; that dealing with claims 6 and 12 is reversed.

Modified.